HYPOTHALAMIC-PITUITARY-ADRENAL AXIS REGULATION OVER THE LIFESPAN: CONTRIBUTION OF DIETARY AND LIFESTYLE FACTORS

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ABSTRACT

A delicate balance exists between the protective effects of adrenal glucocorticoids (GCs) secreted in response to stress and the negative consequences that the excessive production of these hormones may have for numerous systems. GCs are involved in the regulation of the stress response, have effects on feeding and body weight gain and are associated with the acceleration of central nervous system aging. Their production, secretion, and containment are subject to both environmental modulation and individual variation. Neonatal manipulations known to affect the development of the adult hypothalamic-pituitary-adrenal (HPA) response to stress had profound effects on both basal and stress-induced dietary choice, body weight and insulin dynamics. We followed this study with an examination of how the physiological and emotional response to stress can affect diet choices and affective status. Stress had an impact on diet choice and had a strong effect on emotional status but did not affect subjects uniformly. We then explored the reciprocal relationship, i.e. how diet itself can affect the response to stress and found that basal and stress-induced activation of the HPA axis in both young and aged rats is augmented following the feeding of high-fat diets and fat-feeding cause aberrations in glucose-insulin axis. Since aging can be associated with profound changes in HPA axis function, we assessed how dietary habits may contribute to the emergence of the cortisol (F) profile in a population of healthy elderly humans. We found a strong positive relationship between individual F production, feelings of depression and the high fat content in their diets. While dietary habits may have a negative impact on the emergence of an individuals' cortisol profile and on the aging process, we wanted to explore whether a beneficial intervention at mid-life would allow animals to age "successfully" by

reducing glucocorticoid production. Environmental enrichment lowered corticosterone levels and spared animals from the negative effects of glucocorticoids on cognition; environmentally enriched rats had lower overall B, higher glucose and performed as well as young rats on a number of behavioral paradigms designed to assess memory and anxiety. Taken together, these studies suggest that the damaging effects of adrenal glucocorticoid production can be either augmented or avoided by both acute and chronic environmental and dietary interventions imposed early on or late in life.

RESUMÉ

Un fragile équilibre existe, au sein de divers systèmes physiologiques, entre l'action protectrice qu'exerce les hormones stéroïdiennes sécrétées par les surrénales et les conséquences négatives d'une exposition aigu et chronique à ces hormones. Les glucocorticoïdes sécrétés par les glandes surrénales sont impliqués dans la régulation de la réponse au stress et jouent un rôle primordial dans le contrôle de l'appétit et le prise ponderale de poids corporel. La production et la sécrétion de ces hormones stéroïdiennes sont affectées par des variations de certains déterminants environnementaux et individuels (physiques et psychologiques). Il a été démontré que certaines manipulations néonatales affectant le développement de l'axe hypothalamo-hypophyso-surrénalien (HPA *: hypothalamic-pituitary-adrenal*) impliqué dans la réponse au stress pouvaient provoquer chez l'adulte de profonds changements, basal et induits par le stress, dans les comportements alimentaires, le poids corporel et le taux d'insuline. Dans un premier temps, nous avons poursuivi ces travaux et examiné comment la réponse psychologique et physiologique au stress peut affecter les comportements alimentaires et les émotions. Le stress en affectant le comportement alimentaire peut jouer un rôle important sur les émotions. Dans un deuxième temps, afin de mieux comprendre cette relation de réciprocité stress-alimentation sur les états "émotifs" nous avons examiné comment la diète en elle-même peut affecter la réponse au stress. Nos résultats démontrent que l'activation de l'axe HPA, les taux basal et induits par un stress, est augmenté suite à la consommation d'une diète riche en gras chez des rats jeunes ou âgés. Il a été démontré que le vieillissement peut être associé avec de profonde modification du fonctionnement de l'axe HPA. Afin de mieux cerner pourquoi ces modifications surviennent nous avons

examiné, dans une population en santé de personnes âgées, comment les habitudes alimentaires affectent l'émergence de ce profil de sécrétion de la cortisone par les surrénales. Nous avons observé une forte corrélation positive entre la production individuelle de glucocorticoïdes, l'apparition de troubles dépressifs et la consommation d'une diète élevé en gras. Ainsi, les habitudes alimentaires semblent avoir un impact négatif sur l'apparition d'un profil particulier de sécrétion de la cortisone et sur le processus de vieillissement, nous avons donc vérifer si une intervention appropriée à la mi-terme de la vie pourrait favoriser, en réduisant la production de glucocorticoïdes, un vieillissement reussi dans notre modèle animal. Nos résultats démontrent qu'une alimentation adéquate favorise, chez le rat, une diminution du taux de corticostérones et assure une protection contre les effets négatifs des glucocorticoïdes sur le fonctionnement cognitif. Ainsi, nous avons observé que les rats âgés maintenus dans cet environnement enrichit performaient aussi bien que les jeunes rats dans différents tests comportementals utilisés pour mesurer la mémoire et l'anxiéte. En conclusion, les résultats de ce projet de recherche suggèrent que dans de nombreux cas, les changements biologiques dégénératifs (ex. les effets négatif sur la production des glucocorticoïdes par les surrénales) associés au vieillissement peuvent être aggravés ou éliminés par une intervention aigue et chronique, en bas âge ou plus tardivement au cours de la vie, sur le stress environnemental et les comportements alimentaires.

AUTHOR'S NOTE

Contributions of co-authors

The paper by Tannenbaum et al. (1997) entitled "High-fat feeding alters both basal and stress-induced HPA activity in the rat" was a multi-centered effort and was made possible by the dedicated efforts of numerous people. As this work is included in the thesis I wish to comply with the copyright agreements and highlight the involvement of all the authors (please see letter from journal).

I was directly involved in the planning of all experiments, carrying out all the experimental analysis, data handling, conference presentations and writing the manuscript in collaboration with Dr. Michael Meaney, Dr. Gloria Tannenbaum, Dr. David Brindley, Dr. Mary Dallman and Dr. Dawn MacArthur. The basal samplings were carried out in Dr. Tannenbaum's lab, the negative feedback study was done in collaboration with Dr. Dallman's lab and Drs. Brindley and MacArthur were involved in the free fatty acid assays. Shakti Sharma assisted me with some of the radioimmunoassays, and Miss Darlene Francis was involved in the blood sampling.

In addition to the collection of the main data presented in this thesis, I was actively involved in numerous other experiments. I am a co-author on these studies and they include:

Caldji C. **Tannenbaum B.** Sharma S. Francis D. Plotsky PM. Meaney MJ. Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. Proceedings of the National Academy of Sciences of the United States of America. 95(9):5335-40, 1998

Liu D. Diorio J. **Tannenbaum B.** Caldji C. Francis D. Freedman A. Sharma S. Pearson D. Plotsky PM. Meaney MJ. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science. 277(5332):1659-62, 1997

Tannenbaum B. Rowe W. Sharma S. Diorio J. Steverman A. Walker M. Meaney MJ.Dynamic variations in plasma corticosteroid-binding globulin and basal HPA activity following acute stress in adult rats. Journal of Neuroendocrinology. 9(3):163-8, 1997

Meaney MJ. O'Donnell D. Rowe W. **Tannenbaum B.** Steverman A. Walker M. Nair NP. Lupien S. Individual differences in hypothalamic-pituitary-adrenal activity in later life and hippocampal aging. Experimental Gerontology. 30(3-4):229-51, 1995

Meaney MJ. Diorio J. Francis D. LaRocque S. O'Donnell D. Smythe JW. Sharma S. **Tannenbaum B.** Environmental regulation of the development of glucocorticoid receptor systems in the rat forebrain. The role of serotonin. Annals of the New York Academy of Sciences. 746:260-73; discussion 274, 289-93, 1994

Meaney, M.J., **Tannenbaum, B.**, Francis, D., Bhatnagar, S., Shanks, N., Viau, V., and O'Donnell, D. Early environmental programming hypothalamic-pituitary-adrenal responses to stress. Seminars in Neuroscience, 1994, 6, 1-13.

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To find some vacant bench space, you'd only have to ask.
But once you got in the door, one thing became quite true,
That bench space was extended into the hallway and the loo.

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So some worked in the daytime and some worked in the nights.
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LIST OF ABBREVIATIONS

ABBREVIATION	FULL TERM
ACTH	adrencorticotrophin hormone
ACh	acetylcholine
ADX	adrenalectomized
AI	aged cognitively impaired
ALDO	aldosterone
ANG 11	angiotensin 11
AP-1	activator protein-1
ARC	arcuate nucleus
AU	aged cognitively unimpaired
AVP	arginine vasopressin
B	corticosterone
BDNF	brain-derived nerve factor
BN	bombesin
BNST	bed nucleus of the stria terminalis
CARB	carbohydrate
cAMP	cyclic AMP
CBG	corticosteroid binding globulin
CCK	cholecystokinin
CGRP	calcitonin gene related peptide
CMS	chronic mild stress
CNS	central nervous system
CRF/CRH	corticotrophin releasing factor/hormone
CSF	cerebral spinal fluid
DA	dopamine
DEX	dexamethasone
DHEA	dehydroepiandrosterone
DHEA-s	dehydroepiandrosterone-sulfate
DNA	deoxyribonucleic acid
EE	environmental enrichment
EEA	excitatory amino acid
ER	endoplasmic reticulum
ENK	enkephalin
EPI	epinephrine
5-HT	serotonin
5-HIAA	5-hydroxy indole acetic acid
F	cortisol
FA	fatty acid
FFA	free-fatty acid
GABA	γ–aminobutyric acid
GAL	galanin

GC(s)	glucocorticoid(s)
GH	growth hormone
GHRH	growth hormone releasing hormone
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HDL	high-density lipoprotein
HMGCoA reductase	3-hydroxy-3-methylglutarylcoenzyme A reductase
HPA	hypothalamic-pituitary-adrenal
HSP	heat shock protein
HVA	homovanillic acid
icv	intracerebroventricular
IDDM	insulin-dependent diabetes mellitus
IGF	insulin-like growth factor
IL	interleukin
ip	intraperitoneal
ir	immunoreactive
IEG	immediate early genes
LDL	low-density lipoprotein
LH	leutinizing hormone
LHRH	leutinizing hormone releasing hormone
MPOA	medial preoptic area
MpFC	medial prefrontal cortex
MpPVN	medial parvocellular paraventricular nucleus
MR	mineralocorticoid receptor
mRNA	messenger RNA
MRI	magnetic reasonance imaging
NA	noradrenaline
NEFA	non-esterified fatty acid
NGF	nerve growth factor
NIDDM	non-insulin dependent diabetes mellitus
NMDA	n-methyl-d-aspartate
NO	nitric oxide
NPY	neuropeptide Y
NT	neurotensin
NTS	nucleus tractus solitaris
OT	oxytocin
PFC	prefrontal cortex
PHI	peptide-histidine-leucine
PNMT	phenylethanolamine
POMC	pro-opiomelanocortin
PRL	prolactin
PTSD	post-traumatic stress disorder

PVN	paraventricular nucleus
RIA	radioimmunoassay
SCN	suprachiasmatic nucleus
SCP	sterol carrier protein
SFO	subfornical organ
STZ	streptozotocin
TH	tyrosine hydroxylase
TRH	thyrotropin releasing hormone
VBS	visible burrow system
VMH	ventromedial hypothalamus
VIP	vasoactive intestinal peptide
VLDL	very low density lipoprotein
VTA	ventral tegmental area

INTRODUCTION

GENERAL STATEMENT

Previous research in the field of stress and disease suggests that both basal and stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis, the primary regulator of the stress responses, is intricately influenced by a number of variables: these include, but are not limited to, neonatal influences, the metabolic status of the organism, the age of the organism, signals and stimulation from the environment, and the organism's history of trauma. Each of these variables can, in turn, be subject to regulation by the HPA axis itself. Subtle variation in the early neonatal environment, for example, has long-lasting and permanent effects on the development of the HPA axis, and these effects persist well into old age. These effects on the HPA axis are associated with the emergence of age-related pathology, such as cognitive impairments and carbohydrate metabolism, and the extent of both cognitive and metabolic impairment are very much determined by the HPA status of the organism. While the permanence of these effects cannot be underscored, variables imposed as late as mid-life can have striking effects on the aging process. This is critical in our understanding of aging, since it seems that neural aging is more a consequence of lifestyle factors, such as the diet, than a predetermined sequelae of progressive decline. These lifestyle variables, however, do not have uniform effects; their effects are strongly dependent on when they are imposed and for the duration of time that the organism is exposed to them. In the series of studies presented here we are attempting to describe how short and longer-term dietary and environmental manipulations affect the HPA axis throughout life, how the early environment affects later feeding preferences, both of which are largely governed by HPA axis functioning and how both acute and chronic stressors themselves affect food intake. Our studies show that a remarkable amount of flexibility exists across systems where a parallel system can be accessed to compensate for a change in the output of another system, depending on the time and extent that a variable is

imposed. Our data suggest that not only is aging a non-uniform process, it is a process that is subject to plasticity late in life. The early environment has similar effects, whereby subtle variations in rearing during the first three weeks of life can have a permanent impact on the development of feeding patterns and body weight, which may themselves impact the magnitude of glucocorticoid secretion over the lifespan and the development of pathology. Our human studies suggest just that: perhaps one source of variation in cortisol secretion and the associated cognitive impairment in a healthy elderlies is eating patterns. Therefore the interrelationship between diet, lifestyle factors and the HPA axis may be one window into the individual way in which we age.

The Hypothalamic-Pituitary-Adrenal Axis

In mammals, the response to stress remains ill defined. It generally refers to physical or psychological alterations capable of disrupting homeostasis. This "stress response" involves a number of processes comprising what was termed by Selye the "General Adaptation Syndrome" (Selye, 1956). A major neuroendocrine component of this response is mediated by the HPA axis. Various stress inputs converge on the neurons of the hypothalamic paraventricular nucleus (PVN). The PVN neurons that synthesize corticotrophin-releasing-hormone (CRH) (Vale et al., 1981), arginine vasopressin (AVP) and other secretagogues project to the external layer of the median eminence, where they release their products into the portal circulation, resulting in the synthesis and release of adrenocorticotropic hormone (ACTH) from the anterior pituitary corticotrophs. The ACTH, in turn, activates the biosynthesis and release of adrenal glucocorticoids (GCs) from the adrenal cortex, which act broadly throughout the body to mediate changes in processes (eg. metabolic, immune, inflammatory) required for adaptation. Due to their potency and wide range of action, GCs must be maintained at appropriate levels; either too much or too little is deleterious to an organism. This regulation is accomplished through multiple feedback loops operating at pituitary and brain levels.

Paraventricular Nucleus of the Hypothalamus

Neuroanatomy and Neurochemistry

The final common pathway controlling activity of the HPA axis originates in the PVN. This wing-shaped nucleus positioned at the rostral end of the hypothalamus along the dorsal border of the third ventricle can be divided into distinct subterritories on the basis of cytoarchitectural, cytochemical and connectional features (Swanson and Sawchenko, 1983). The PVN can be separated into magnocellular and parvicellular divisions. Magnocellular neurons are large neurosecretory cells that primarily synthesize AVP or oxytocin (OT), in addition to a number of neuropeptides, project to the posterior pituitary, and release their products into the peripheral circulation (Cullinan et al., 1995). The classic role of AVP released from magnocellular neurons is in maintenance of water and salt balance, while OT released from the posterior pituitary is critical for lactation and parturition. Parvicellular components of the PVN can be divided into five distinct subregions: the periventricular region, anterior parvicellular region, medial parvicellular region (further divided into dorsal and ventral components), dorsal parvicellular region and lateral parvicellular region (Swanson and Kuypers, 1980). Neurons located mainly within the dorsal portion of the medial parvicellular region, which synthesize CRH, project to the external lamina of the median eminence and release CRH into the hypophyseal portal vasculature, comprise the origin of the final common pathway for ACTH release. The periventricular and dorsal medial parvicellular regions contain neurons that synthesize somatostatin, growth hormone releasing hormone (GHRH), thyrotropin-releasing hormone (TRH) and dopamine; these neurons also project to the external lamina of the median eminence and control the release of growth hormone (GH), thyrotropin, and prolactin (PRL) from the anterior pituitary (Swanson et al., 1986). The anterior, ventral medial parvicellular and lateral parvicellular regions of the PVN contain cells that project to the intermediolateral column of the spinal cord and/or dorsal vagal complex and other parasympathetic cell

groups, while dorsal parvicellular neurons project to the intermediolateral column of the spinal cord; the descending projections of these parvicellular neurons impart an integral function in the regulation of the autonomic nervous system. Coupled with the endocrine regulation afforded by the magnocellular and neuroendocrine parvicellular neurons, the PVN is thus considered a critical locus for integration and regulation of pathways concerned with the maintenance of homeostasis.

Release of ACTH

CRH is acknowledged as the primary ACTH secretagogue due to its potent intrinsic ACTH releasing activity (Vale et al., 1983). Pharmacological blockade of CRH can eliminate the ACTH response to various stress conditions (Plotsky et al., 1985a, b). A number of additional secretagogues present within the dorsal medial parvicellular region of the PVN have been found to be co-stored with CRH. AVP has been detected in 50% of CRH neurons in male rats treated with colchicine (Whitnall, 1988). Terminals of CRH neurons are segregated into vasopressin-rich and vasopressin-deficient subpopulations that are differentially regulated by GCs and stress (Whitnall et al., 1987a;b;Whitnall, 1989; deGoeij et al., 1991). In addition to AVP, cholecystokinin (CCK), galanin, vasoactive intestinal peptide (VIP), GABA, angiotensin II, enkephalin, neurotensin (NT) and peptide histidine-isoleucine (PHI) have all been found to be co-localized in CRH neurons. AVP has been shown to have weak effects on ACTH release, but can greatly potentiate the ability of CRH to stimulate ACTH secretion (Vale et al., 1983; Plotsky et al., 1985; Rivier and Vale, 1983). Weak activity of CCK, VIP, PHI and angiotensin II have also been found on ACTH release (Tilders et al., 1984; Antoni, 1986; Mezey et al., 1986; Watanabe and Orth, 1988).

Different categories of stressors may target distinct populations of hypophysiotropic neurons through different projections. Plotsky et al. (1985a,b) reported that hemorrhage causes an increase in portal levels of CRH and AVP, while insulin-induced

hypoglycemia causes an increase in portal levels of AVP only. Ether stress but not cold or swim stress has been shown to cause an increase in enkephalin mRNA levels within the mpPVN (Watts, 1991; Ceccatelli and Orazzo, 1993). These data suggest that there is a precise regulation of the ACTH secretagogue signal reaching the anterior pituitary corticotrophs. Magnocellular neurosecretory neurons represent another potential source of ACTH-releasing activity. A largely magnocellular origin of AVP and OT in the hypohyseal portal blood has been reported (Antoni et al., 1990) and OT, similar to AVP, has been shown to have weak intrinsic ACTH-releasing activity and can potentiate the ability of CRH to stimulate ACTH secretion (Linke et al., 1992). A role for magnocellular neuropeptides in the regulation of ACTH secretion has begun to emerge in recent studies: inhibition of magnocellular neurons partially abolishes the ACTH response to specific stressors (Dohanics et al., 1991). The role of magnocellular peptides contributing to stress-induced ACTH secretion remains to be clearly defined.

Afferent Regulation of the PVN

PVN neurons are known to receive a diverse set of inputs from the brainstem, the limbic telencephelon, the hypothalamus itself and the circumventricular regions (Cullinan et al., 1995).

a) Brainstem Pathways

An enhancement of catecholaminergic activity following exposure to different forms of stress has long been established. The brainstem noradrenergic and adrenergic inputs to the PVN have been mapped in detail and ultrastructural evidence has confirmed noradrenergic and adrenergic synapses on CRH neurons (Cunningham and Sawchenko, 1988; Cunningham et al., 1990). The noradrenergic innervation of the PVN emanates from the A1 cell group of the ventrolateral medulla, the A2 cell group, corresponding to the nucleus of the NTS, and the A6 cell group of the locus coeruleus. However, while the A1 cell group appears to project primarily to the magnocellular portions of the PVN

(Cunningham et al., 1990), it is the A2 catecholaminergic cell group that provides the CRH zone of the PVN along with its major noradrenergic innervation, along with a minor contribution from the locus coeruleus (A6) (Cunningham and Sawchenko, 1988). These PVN-projecting catecholaminergic inputs also display a number of peptides that are colocalized in these cells. NPY is found in most adrenergic neurons that project to the PVN as well as in a subpopulation of PVN-projecting neurons in the A1 region (Sawchenko et al., 1985) and galanin is colocalized in PVN-projecting neurons within A1 and A6 (Levin et al., 1987). Adrenaline can have stimulatory effects on the HPA axis (Plotsky, 1987), however evidence suggesting an inhibitory role has also been reported (Mezey et al., 1984). While catecholaminergic neurons appear to be important in the early activation of the HPA axis, at least for several forms of stress, the question of where such influences are exerted remains partly unanswered. For example, the locus coeruleus is one of the most stress-responsive regions in the brain (Cullinan et al., 1995) and has been implicated in the regulation of HPA responses to hemorrhage (Gann et al, 1977). However, the locus coeruleus has quite limited direct input to the PVN region and thus might exert HPA effects through its dense innervation of central limbic structures (Herman and Cullinan, 1997). The question of whether these catecholaminergic influences are exerted at the level of the PVN or the median eminence or both and how receptors may stimulate the activity (although alpha-adrenoreceptor involvement in the PVN has been shown (Plotsky et al., 1989) remains.

The role of serotonin (5-HT) in HPA regulation has been debated; some studies indicate excitatory actions on 5-HT on ACTH and B release (Feldman et al., 1987), whereas others indicate concentration-dependent facilitatory and inhibitory effects on HPA tone (Korte et al., 1991; Welch et al., 1993). Direct 5-HT innervation of the PVN is somewhat limited, suggesting the potential for indirect actions by way of other stress pathways (Sawchenko et al., 1983). There is a modest projection to the CRH zone of the PVN, originating from the brainstem serotonergic cell groups B7-B9 (Sawchenko et al., 1983)

and Liposits group (1987) has found evidence for synaptic contact between serotonergic terminals and CRH neurons. It has been suggested that the serotonergic pathways involved in HPA activation may be stressor-specific: while depletion of hypothalamic serotonin appears to reduce the adrenocortical response to photic stimuli, the lesions have no effect on the adrenocortical response to ether, restraint, hypertonic saline administration or insulin-induced hypoglycemia (Cullinan et al., 1995).

Finally, Sawchenco and colleagues (reviewed in Sawchenko, 1991) have identified a number of mesencephalic and pontine cell groups that may act to relay sensory information to the PVN. These include the posterior intralaminar and peripeduncular nuclei that relay auditory information (LeDoux et al., 1985), periaqueductal gray and pontine central gray that relay sensory and nocieceptive information and the pedunculopontine nucleus and laterodorsal tegmental nucleus that may relay visual and auditory information (Cullinan et al., 1995). These cell groups are largely cholinergic and the proposed pathway is consistent with previous evidence suggesting stimulatory effects of acetylcholine on CRH secretion and ACTH release (Ohmori et al., 1995). However, anatomical studies again suggest very sparse cholinergic innervation of the PVN proper, suggesting that cholinergic actions might be relayed by hypothalamic local circuit neurons (Ruggiero et al., 1990).

b) Limbic Inputs

Numerous studies have indicated that HPA activity can be influenced by portions of the limbic telencephalon, including the hippocampus, medial prefrontal cortex, septum and amygdala (Cullinan et al., 1995). The amygdala is known to prompt behavioral and cardiovascular responses to stress (Davis, 1992). Damage to the amygdala has been shown to decrease ACTH and B secretion following adrenalectomy (Allen and Allen, 1975). More detailed analyses suggest that excitatory effects of the amygdala on HPA function are mediated by the central, medial and cortical amygdaloid nuclei. Stimulation of the medial or cortical amygdaloid nuclei elicits B secretion (Dunn and Whitener, 1986). Ablation of the

medial or central amygdaloid nuclei block HPA responses to acoustic and photic stimulation (Feldman et al., 1994); other studies indicate that lesions of the central nucleus of the amygdala decrease ACTH and B responses to restraint and fear conditioning (Van de Kar et al., 1991). However, medial and central amygdaloid lesions do not block HPA responsiveness to ether (Feldman et al., 1994), providing evidence for stressor-specificity in amygdaloid stress paths.

The bed nucleus of the stria terminalis (BST) may also convey excitation of the HPA axis. This limbic forebrain structure links regions such as the amygdala and hippocampus with hypothalamic and brainstem regions controlling vital homeostatic functions (Cullinan et al., 1993). Specific ablation of lateral divisions of this region decreases expression of CRH mRNA in the PVN (Herman et al., 1994) and attenuates B secretion induced by conditioned fear (Gray et al., 1993), whereas stimulation of the lateral BST increases B secretion (Dunn, 1987). These cell groups are considered by many to be extensions of the central amygdaloid nucleus. This notion is supported by similar effects of lesions of the central amygdaloid nucleus and lateral BST on the stress axis. Finally, recent experiments have suggested the existence of a hippocampal-BST-PVN circuit (Cullinan et al., 1995) and that a significant proportion of the BST projection to the PVN may be GABAergic. Such a pathway might explain the fact that hippocampal stimulation results in a reduction in circulating B and that ablation of its major efferent pathways leads to B hypersecretion and induction of CRH mRNA in the mpPVN (Herman et al., 1989).

c) Hypothalamic Inputs

It has been suggested that the PVN receives input from a number of hypothalamic sources; at least some PVN-projecting neurons can be localized within virtually all hypothalamic regions (Swanson, 1987). The PVN has been shown to receive a prominent GABAergic innervation, and GABA-containing terminals have been confirmed to contact CRH neurons (Decavel et al., 1990). Hypothalamic GABAergic inputs into the PVN may

serve to relay limbic inputs to CRH neurons (Cullinan et al., 1995). Support for GABA-mediated inhibition of HPA activity has come from in vitro studies, as well as in vivo experiments demonstrating GABAergic inhibition of CRH release and ACTH secretion (Plotsky et al., 1987; Caldji et al., unpublished observations). It has also been shown that glutamate- and possibly Ah-containing neurons play a role in the excitation of the PVN. Injection of glutamate into the PVN activates neurosecretory neurons (Tasker and Dudek, 1993). Furthermore, parvocellular PVN neurons express NMDA, kainate and AMPA receptors (Van den Pol et al., 1994), and appear to receive synaptic input from glutamate-containing neurons (Decavel and Van den Pol, 1992). Acetylcholine is known to enhance CRH release in explant cultures and to increase ACTH release and expression of CRH mRNA following microinjection into the PVN (Ohmori et al., 1995). Anatomical studies, however, suggest very sparse cholinergic innervation of the PVN proper, suggesting that cholinergic actions might be relayed by hypothalamic local circuit neurons (Ruggiero et al., 1990).

d) Circumventricular Inputs

This class of efferents include: the subfornical organ (SFO), the median preoptic nucleus and the organum vasculosum of the lamina terminalis. These structures are known to be involved with water balance, and while their projections appear to principally affect magnocellular components of the PVN, recent anatomical and physiological data suggest that parvicellular elements are also influenced (Kovacs and Sawchenko, 1993). These paths are thought to mediate the effects of blood-borne angiotensin II on the HPA axis: the SFO has been shown to be important in sensing circulating AII (Simpson, 1981). AII has been localized in the projections from SFO and the median preoptic nucleus to the PVN and the SFO sends an additional AII-containing projection to the median preoptic nucleus (Lind et al., 1984).

Adrenal Gland

The HPA axis is primarily responsible for the release of glucocorticoids (GCs) into the systemic circulation. GCs are known to be key players in the organismic response to stress. Upon release, these hormones bind to high-affinity cytosolic receptor molecules and are translocated to the cell nucleus, where the receptor-ligand complexes dimerize and bind to DNA (Drouin et al., 1992). At the DNA level the ligand-receptor complexes act to modulate the transcription of a staggering variety of genes (Herman, 1993). By way of this genomic effect, GCs have the capacity to interact with multiple systems at all levels of the body. GCs initiate a number of adaptive responses from multiple organ systems. The functions of released GCs include the mobilization of glucose from the liver, an increase in cardiovascular tone, altered immune functions and inhibition of nonessential endocrine systems (Munck et al., 1984). Actions of the GCs are largely catabolic, serving to consume resources. Thus, while being adaptive during periods of active coping is required, pronounced GC secretion is clearly maladaptive when prolonged or exaggerated.

General Physiological Functions of the GCs.

The adrenal cortex produces three principal categories of steroid hormones. The mineralocorticoids, of which aldosterone is most important, regulate renal sodium retention and thus are key components in regulating sodium, potassium, blood pressure, and intravascular volume. Mineralocorticoid excess can cause hypertension and hypokalemia; mineralocorticoid deficiency can cause hyponatremia, hyperkalemia, hypotension and shock. The glucocorticoids include cortisol (F) , the primary one in humans and corticosterone (B), primary in rats. They are named for the role in maintaining serum glucose and regulating carbohydrate metabolism, and also play key regulatory roles in a wide variety of physiologic processes, including feeding, development, growth, immune responses, cardiovascular function, and responses to stress. The adrenal cortex secretes

over 50 steroids; while many of them are precursors or metabolites of the principal hormones, most have some biological activity.

Anatomy of the adrenal glands

The adrenal glands are extraperitoneal at the upper poles of the kidneys. They enlarge in response to ACTH and becomes smaller when ACTH production decreases (Felig et al., 1995). Blood is supplied into the adrenal by several short arteries that are terminal branches of the inferior phrenic artery. Arterial blood enters a sinusoidal circulation in the cortex and drains towards the medulla so that medullary chromaffin cells are bathed in a high concentration of steroid hormones. The innervation of the adrenal gland is autonomic (Netter, 1965). Sympathetic preganglionic fibres are axons of cells in the lower thoracic and upper lumbar segments, whereas the parasympathetic fibres come from the celiac branch of the posterior vagal trunk. Most of the nerves are contained in an adrenal plexus along the medial border of the adrenal gland, enter it as bundles near the hilus and run through the cortex to the medulla. This innervation may participate in the control of adrenocortical growth and steroid secretion, and an afferent neural pathway from the adrenal to the hypothalamus mediates stress-induced feedback inhibition of ACTH secretion (Dallman, 1985).

In adults the cortex makes up about 90 percent of the adrenal and surrounds the centrally located medulla. The cortex consists of three distinct zones: the zonae glomerulosa, fasciculata, and reticularis. The zona glomerulosa produces aldosterone but not cortisol; the zona fasciculata produces cortisol but not aldosterone and the zona reticularis also produces cortisol (albeit at a much slower rate) and the major adrenal androgens dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEA-S) (Felig et al., 1995). ACTH has a dramatic effect on the adrenal: within 2-3 minutes of ACTH administration, adrenal blood flow increases and cortisol is released. Within hours, adrenal weight increases. Prolonged ACTH stimulation causes both adrenal hypertrophy and

hyperplasia, while ACTH deficiency can lead to atrophy of the zona fasciculata and reticularis.

Synthesis and Release of GCs

All steroid hormones are derived from pregnenolone. Pregnenolone and all its naturally occurring mineralocorticoid and glucocorticoid products contain 21 carbon atoms and hence are termed C-21 steroids. With the exception of estrogens, all steroid hormones have a single unsaturated carbon-carbon double bond. The pathway of synthesis from pregnenolone to aldosterone is the mineralocorticoid pathway; this is found almost exclusively in the zona glomerulosa. All these compounds have 21 carbon atoms. The pathway from 17-hydroxypregnolone to cortisol is the glucocorticoid pathway, found in the zona fasciculata and, to a lesser extent, the zona reticularis. These are all 17-hydroxy C-21 steroids.

Cholesterol is the precursor of steroid hormones. The adrenal readily synthesizes cholesterol from acetyl-CoA in response to trophic stimulation by ACTH (Brown et al., 1979). The activity of 3-hydroxy-3-methylglutarylcoenzyme A reductase (HMGCoA reductase), the rate-limiting enzyme in cholesterol synthesis, generally parallels steroidogenic activity. HMGCoA reductase is inactivated by phosphorylation and is activated by dephosphorylation in response to intracellular cAMP elicited by the binding of ACTH to its receptor. About 80 percent of the cholesterol used for steroid hormone synthesis derives from dietary cholesterol transported in the plasma as low-density lipoprotein (LDL) particles synthesized in the liver (Brown et al., 1979). Adrenal uptake of LDL is regulated coordinately with steroid synthesis. LDL uptake principally entails receptor-mediated endocytosis; less than 10 percent of LDL enters the cell by a receptor-independent mechanism (Brown et al., 1979).

Conversion of cholesterol to pregnelonone is a complex process, requiring many steps. First, cholesterol fluxes into the adrenal mitochondria. Cholesterol is then stored as

cholesterol esters in lipid droplets that bud off from the endoplasmic reticulum (ER). The cleavage of the side-chain of cholesterol requires the actions of three proteins, including cytochrome P450. This takes place in the mitochondria and is considered the rate limiting step in steroidogenesis (Gower, 1975). Pregnenolone transfers out of the mitochondria to the ER where it is converted to progesterone and then deoxycorticosterone. Deoxycorticosterone returns to the mitochondria and is converted by 11-B hydroxylase to corticosterone (B). B then travels out of the mitochondria to the cytosol and is secreted into the general circulation (Felig et al., 1995).

ACTH or other tissue-specific tropic hormones stimulate esterases and inhibit synthetases, thus increasing the availability of free cholesterol for steroid hormone synthesis (Jefcoate et al., 1986). ACTH rapidly facilitates the transport of cholesterol across the mitochondrial membrane, the binding of cholesterol to P450scc (scc refers to the side chain cleavage of cholesterol), and the release of newly synthesized pregnenolone from mitochondria. ACTH acutely increases cortisol synthesis and release within 2-3 minutes. When ACTH concentrations fall, steroidogenesis decreases rapidly. ACTH receptors are found on the surface of adrenal cortical cells, where they bind ACTH with a K_d of about 10^{-9} *M* (Catalano et al., 1986). Binding of ACTH to its receptor activates adenylate cyclase which increases extracellular levels of cAMP, which in turn activates a number of protein kinases. Prolonged stimulation with ACTH increases adrenal protein and DNA synthesis and net growth (Dallman, 1985). ACTH chronically increases steroidogenesis by promoting the transcription of genes for steroidogenic enzymes. Physiologic concentrations of ACTH also directly stimulate the synthesis and accumulation of insulin-like growth factor II (IGF-II) mRNA and IGF-II peptide. ACTH also chronically stimulates LDL uptake and metabolism and the synthesis and accumulation of LDL receptor and HMGCoA reductase and their mRNAs. Furthermore, ACTH also stimulates cholesterol esterase (Suckling et al., 1983), sterol carrier protein (SCP) (Conneeley et al., 1984) and endozepine (Yanagibashi et al., 1988) so that it has general tropic effects on all

known early steps in steroidogenesis. A role for newly synthesized phospholipids in mediating the chronic actions of ACTH has been proposed. One mechanism of action might be related to promoting the flux of free cholesterol across the mitochondrial membranes to P450scc (Farese and Sabir, 1980).

Factors other than ACTH probably regulate B synthesis and/or release in normal animals, either directly or by modulating the effects of ACTH. A direct effect of GCs on the adrenal cortex has also been proposed. GCs can inhibit steroidogenesis in primary cultures of human adrenal cells in vitro; however, such direct feedback probably plays a minor role in human physiology, as the hypercortisolism found in syndromes of ACTH excess does not effectively reduce steroidogenesis, and the administration of dexamethasone (DEX) does not inhibit ACTH-induced cortisol secretion (Keller-Wood and Dallman, 1984). CRH of adrenal medullary origin can modulate adrenocortical release of B. Van Oers and colleagues (1992) found that in rats administered CRH antibodies, exogenous doses of ACTH produced lower release of B than in animals not treated with CRH antibodies (Van Oers et al., 1992). In addition, they found that addition of CRH and ACTH seem to have synergistic effects on the release of B and the effects of CRH on the release of B seem to be a result of alterations in blood flow through the adrenal (Van Oers et al., 1992). Studies on perfused pig adrenals have demonstrated a release of vasoactive intestinal peptide (VIP) in response to splancnic nerve stimulation, and have further shown that administration of VIP causes an increase in both aldosterone and cortisol secretion, in the absence of ACTH (Ehrhart-Bornstien et al., 1991). Further studies have shown that long-term (7 days) administration of VIP to intact rats stimulates the growth and steroidogenic capacity of the zona glomerulosa, with no apparent effect on corticosterone secretion and the zona fasciculata (Mazzochi et al., 1987). It has been suggested that the discrepancies between the different responses to VIP may be explained if VIP is exerting an indirect effect on adrenocortical cells, possibly by acting on chrommaffin cells in the outer part of the gland (Vinson et al., 1994). Calcitonin-gene-related-peptide (CGRP) administration to the

perfused rat adrenal results in an increase in perfusion medium flow rate through the gland, accompanied by an increase in B (Hinson and Vinson, 1990). Finally, there are interactions between GCs and catecholamines from the adrenal medulla. *In vitro* epinephrine (EPI) can increase the synthesis of enzymes involved in steroid biosynthesis (Ehrhart-Bornstein et al., 1991) and perfusion of adrenals with catecholamines results in the release of GCs (Bornstein et al., 1990). There are also chromaffin cells in the adrenal cortex, and these cells have been shown to release their exocytotic products onto cortical cells (Bornstein and Ehrhart-Bornstein, 1992), suggesting that adrenal medulla catecholamines can release GCs from the adrenal cortex.

Physical state of steroids in plasma-CBG and albumin

Cortisol and corticosterone released by the adrenal gland is free. However, approximately 90-97% of the circulating cortisol is bound by plasma proteins (Westphal, 1971). About 90% of this binding is with corticosteroid-binding-globulin (CBG, also termed transcortin) which binds cortisol specifically and with high affinity; a lesser quantity is bound by albumin and a negligible amount by other plasma proteins (Felig et al., 1995). CBG is produced primarily in the liver but has also been found in brain, kidney, lung and muscle (Ballard, 1979; Hammond, 1990). Human CBG is a 383-amino acid glycoprotein with a molecular weight of about 58,000 (Hammond, 1990). Rat CBG is also produced in the liver, contains 374 amino acids and has a molecular weight of 42,196 (Smith and Hammond, 1989).

CBG levels in plasma are subject to a number of influences. Circulating GCs at supraphysiologic concentrations decrease CBG concentrations (Rosner, 1991). Following adrenalectomy, rats exhibit increased circulating CBG levels (Dallman et al., 1987). Dallman and colleagues (1987) have also shown that there is a negative correlation between circulating levels of B and of CBG in adrenalectomized (ADX-ed) rats replaced with B pellets that produce circulating levels between 0-15 μg/dl, providing further evidence that B

is able to control levels of its binding globulin. Estrogens increase CBG levels, and elevated concentrations are seen in pregnancy (Hammond, 1990). Thyroid hormones increase CBG synthesis; CBG levels are reduced in hypothyroidism and increased in hyperthyroidism (Westphal, 1971). Inflammation results in decreased levels of CBG at the inflammatory site (Hammond, 1990). Neutrophils at the site of inflammation release a group of proteases including elastase. Elastase can cleave CBG, thereby releasing B. As a result, CBG levels decrease and B levels increase at the site of inflammation thereby enhancing the anti-inflammatory actions of B (Hammond, 1990). Consumption of a high carbohydrate diet can decrease CBG by about 20%. Both acute and chronic stressors have direct modulatory roles on plasma CBG levels (Tannenbaum et al., 1997).

A number of theories have been put forward describing the role of CBG in plasma regulation of B. One theory is that these proteins serve as a reservoir to sequester steroids in an inactive form, thus influencing the availability of steroids to tissues, their receptors, and steroid-metabolizing enzymes. However these plasma-binding proteins have not been proved to be essential for transporting steroids in the circulation, as these steroids are sufficiently water soluble at biologically active concentrations (Ballard, 1979). The steroids are inactive when bound by plasma protein (Ballard, 1979) and B is not bound to CBG when it enters the cell. Thus as free and protein-bound steroid circulate through the various tissues, the free steroid binds specifically to receptors, steroid-metabolizing enzymes and other proteins and binds nonspecifically to a number of cellular components. The dissociated steroid is then free for further tissue uptake. Since the capillary transit time though tissues such as the liver is slower (around 5 seconds) than it is in tissues such as brain (around 1 second), the former tissues can extract a larger proportion of the cortisol (Partridge, 1981). In fact, brain uptake of B in the rat approximates the non-CBG-bound steroid, whereas in the liver it exceeds this fraction by three times and includes CBG-bound steroid (Partridge et al., 1983).

Metabolism and excretion of GCs

The hydrophobic steroids are filtered by the kidney but are actively reabsorbed and are therefore not excreted into the urine (Peterson, 1971). Thus steroids undergo enzymatic modifications that transform them into inactive substances with increased water solubility. The liver is the major site of metabolic conversion (Peterson, 1971). Cortisol is cleared with a half time of 80 to 120 minutes. Some of the metabolic conversions of cortisol include conjugation with glucuronide or sulphate groups; corticosterone has a shorter half time of disappearance and more of the metabolites are excreted into the gut (Monder and Bradlow, 1980).

Corticosteroid Receptors

Uptake and retention of ^{3}H steroid hormones by a cell is the result of binding of the hormone by intracellular receptors, that end up in the cell nuclear compartment, where these receptors bind to specific nucleotide sequences on DNA known as "hormone responsive elements" or "HRE's" (Miner and Yamamoto, 1991). Adrenal steroid receptors are found throughout the nervous system in virtually every cell type of the brain and that they mediate many neurochemical and behavioral actions (McEwen et al., 1986; De Kloet et al., 1998). Further, the uptake and retention of adrenal steroids by the hippocampus, first found in the rat, has been demonstrated in species as divergent as the duck and the rhesus monkey and this seems to be a widespread and evolutionarily stable trait of the hippocampal region (McEwen et al., 1986).

Corticosteroid receptors were originally identified in classical steroid target tissues in the periphery. *In vitro* binding studies revealed that aldosterone exhibited binding to a low-affinity and high-affinity site in the kidney (Rousseau et al., 1972). The high affinity site exhibited a higher preference for aldosterone than B or DEX and was thought to mediate classical mineralocorticoid actions (control of sodium balance) of aldosterone, and thus termed mineralocorticoid receptor (MR). The low-affinity site exhibited preference

for DEX and was similar to the site described in the liver, the traditional site for the glucocorticoid actions of B and, thus termed the glucocorticoid receptor (GR).

GRs and MRs mediate B actions on the brain. GRs occur everywhere in the brain but are most abundant in hypothalamic CRH neurons and pituitary corticotrophs (De Kloet et al., 1998). Aldosterone-selective MRs resembling those in the kidney are expressed at hypothalamic sites involved in the regulation of salt appetite and autonomic outflow (McEwen et al., 1986). The highest MR expression is in the hippocampus but the aldosterone selectivity is lost here. Since these apparently "nonselective" MRs bind B with high affinity (10-fold higher than co-localized GRs), hippocampal MRs will respond strongly to B (McEwen et al., 1968; Veldhuis et al., 1982). B in the hippocampus, then, serves to activate two signaling pathways via MR and GR (Reul and De Kloet, 1985).

De Kloet and Reul (1987) postulated that "tonic influences of B are exerted via hippocampal MRs, while the additional occupancy of GRs with higher levels of B mediates feedback actions aimed to restore disturbances in homeostasis." Thus, MRs are involved in the maintenance of the stress system activity while GR (in coordination with MR) mediate steroid control of recovery from stress. There is an immense amount of corticosteroid receptor diversity in the brain and pituitary, which will be reviewed in detail.

The early studies with tracer amounts of B had identified MRs rather than GRs in the hippocampus, since the tracer dose of B was too low to detect GR (Reul and De Kloet, 1985; Coirini et al., 1985). This became apparent when binding properties of MRs and GRs were studied in the cytosol, using selective GR ligands (RU 28362) that allowed discrimination between the two receptor types (Moguilewsky and Raynaud, 1980). It was found that MRs bind B with an affinity 10-fold higher (dissociation constant or K_d 0.5 nM), while the K_d for GR was between 2.5 and 5 nM. MR and GR are both present in dentate gyrus neurons and CA1 cells; CA3 cells mainly express MR. Subsequently it was shown that low basal B levels predominantly occupy MR. GR can be activated additionally

to MR only when B levels are high, i.e. at the circadian peak and during stress (Reul and De Kloet, 1985; Reul et al., 1990). MRs prevail in limbic brain areas (CA1 and CA3 subfields of the hippocampus, dentate gyrus and lateral septum), while GRs are widely distributed in neurons and glial cells, with highest concentrations in the dentate gyrus, lateral septum, nucleus tractus solitarus, amygdala, and locus coeruleus.

The distribution of GRs in the brain as measured by binding assays and autoradiography is paralleled by the distribution of GR immunoreactivity and GR mRNA. Fuxe and colleagues (1985) found high densities of immunoreactive GRs in the parvocellular PVN and CA1 and CA3 subfields of the hippocampus, amygdala and thalamus. Moderate densities were observed in the thalamus and various layers of the cerebral cortex. Brainstem nuclei such as the NTS, the raphe nuclei and the catecholaminergic and dopaminergic cell groups exhibited strong GR immunoreactivity. The distribution of GR mRNA is similar to that found for GR based on receptor binding studies. Sousa et al. (1989) found that GR mRNA was highly expressed in the CA1 and CA2 subfields of the hippocampus and PVN, moderately expressed in the thalamus, olfactory bulb and cerebral cortex. Lower levels of GR mRNA were detected in cerebellum and in the brainstem.

In terms of MR, Arriza et al. (1988) found that the heaviest labeling, using *in situ* hybridization was found in all subfields of the hippocampus and throughout most of the septum. Lower amounts of labeling were detected in the amygdala and hypothalamus. Heavy labeling was found in the NTS in the brainstem, while more modest amounts were found in the raphe nuclei and locus coeruleus. These data suggest that the protein distribution of GR and MR are similar to the mRNA distribution and that their patterns of distribution are distinct, suggesting differential roles for the two receptors in the regulation of HPA function.

Negative Feedback

The HPA axis is tightly controlled to produce rapid optimal GC responses that can be promptly terminated upon the cessation of stress. This regulation is thought to be achieved through GCs acting via multiple inhibitory feedback loops involving pituitary, hypothalamic, and suprahypothalamic sites (Keller-Wood and Dallman, 1984). The effects of the GCs are mediated by MR and GR, with the low-affinity GR being affected by stress-induced GC secretion and the higher affinity MR being involved with basal HPA tone and diurnal GC rhythms (De Kloet and Reul, 1987).

Negative feedback control of the HPA axis can be considered a servocontrol mechanism operating within a closed-loop system (Keller-Wood and Dallman, 1984). A comparator, located somewhere in the brain, generates an error signal based on detection of the relative concentrations of circulating GCs and strength or pattern of afferent input (whether under basal or stress conditions). The comparator can directly determine both these factors or integrate information on these two factors from a number of sources. The error signal that is generated determines the output of the system, in this case ACTH and, therefore, GC release.

Three experimental approaches have been used to explore the brain feedback sites of GCs: 1) replacement of ADX rats with receptor agonists; 2) pharmacological inhibition of individual receptor types in adrenal intact rats using one more or less selective antagonist and 3) correlative evidence between receptor properties and HPA dynamics (De Kloet et al., 1998). ADX leads to an increase in CRH and particularly AVP mRNA and peptide levels in the PVN and in the external layer of the median eminence (DeGoeij et al., 1992). Basal ACTH levels are dramatically elevated. Levels of 150-300 nM B, occupying both types of receptors and achieved with subcutaneous B implants, efficiently suppresses AVP and CRH expression and release into portal blood (Sapolsky et al., 1990). Implants of naturally occurring and synthetic GCs near the PVN act similarly (Kovacs and Sawchenko, 1996).

The rise in basal trough ACTH is prevented by chronic replacement with very low amounts of exogenous B; under these conditions, B also suppresses the ADX-induced synthesis of AVP, while CRH is not affected by either treatment. The IC_{50} of B suppression is about 0.5 nM in terms of circulating free B, in the range of the MR K_d value. Accordingly MR mediates the proactive feedback mode of B involved in maintenance of basal HPA activity (DeKloet et al. 1998). At the circadian peak, much higher levels of exogenous B are required, and half-maximal suppression is achieved by a free concentration of about 5 nM close to the $K_{d\ of}$ GRs (Reul and DeKloet, 1985). However, exclusive activation of GR is insufficient to suppress the circadian peak, and MR activation appears to be indispensable (Bradbury et al., 1994). The corticosteroid concentration does not need to be continuously high, in that an episodic rise in GC levels by injection or ingestion via normal evening drink is sufficient to occupy both receptor types and to maintain ACTH levels with small amplitude changes over the 24-h period (Bradbury et al., 1991).

These GR-mediated effects observed after exogenous GCs thus are also involved in the maintenance of HPA activity. An interesting paradox is that exogenous B suppresses subsequent stress-induced ACTH levels whereas similar levels of the steroid attained after a first stress do not (Dallman et al., 1992). Stress evokes a local transient condition of steroid resistance in the PVN; the elevated B facilitates the termination of the HPA response to stress, and various temporal (fast, intermediate and slow feedback) domains have been distinguished (Keller-Wood and Dallman, 1984). These GR-mediated effects triggered in response to stress represent the reactive mode of feedback operation (DeKloet et al., 1998).

Time Domains of Glucocorticoid Negative Feedback

Corticosteroid feedback inhibition of ACTH secretion appears to operate in at least three time domains: fast (within seconds to minutes), delayed (over 2-10 hours) and slow (over hours to days) (Keller-Wood and Dallman, 1984). Fast, rate-sensitive feedback, as postulated by Dallman and Yates (1969), occurred when injection of B into rats inhibited the

B response to histamine administration if the injection preceded the histamine administration by 15 seconds or 5 minutes, but not if the B was injected 15 minutes before or 2 minutes after the histamine administration. They proposed that there is a rapid inhibitory effect of B that occurs while plasma concentrations of the hormone are increasing. It was subsequently determined that the rate of increase of B necessary to produce this inhibition was at least 1.3 μg/dl/min (Jones et al., 1972; Abe and Critchlow, 1977). GC feedback inhibition has rapid effects on stimulated CRF and ACTH secretion (Vale and Rivier, 1977). The rapidity of the effect suggests that protein synthesis is not involved and that the effect is on hormone release and not synthesis. This rapid inhibition is not affected by pretreatment of perifused pituitaries with cyclohexamide, therefore the fast feedback action of B does not require protein synthesis (Widmaier and Dallman, 1983).

The second time domain of negative feedback is delayed, intermediate feedback. Dallman and Yates showed that pulses (1969) or infusions of B beginning 120 minutes before histamine injection inhibited the endogenous B response to histamine. However, infusions beginning 45, 19 or 10 minutes before the injection did not inhibit the response. They therefore hypothesized that there is a delayed feedback effect of corticosteroids which is independent of circulating corticosteroid levels at the time of stress, and which requires at least 45 minutes but less than 120 minutes to develop (Keller-Wood and Dallman, 1984). This kind of feedback is determined by the level of steroid achieved or the total dose of steroid administered (Jones et al., 1974). A single dose of DEX can produce inhibition of ACTH and B release from 2 to 4 hours later, and the inhibition produced by infusions of steroids are dependent on the length of the infusion period. It appears that intermediate corticosteroid feedback inhibits ACTH release but not synthesis (Keller-Wood and Dallman, 1984).

Corticosteroid feedback inhibition that results from constant corticosteroid exposure for 12 or more hours has been termed "delayed, slow feedback" (Keller-Wood and

Dallman, 1984). Slow feedback appears after prolonged periods of high plasma or medium concentrations of B or in pathological conditions. Anterior pituitary POMC and ACTH release and synthesis are inhibited by slow feedback (Engeland et al., 1975). In sum, fast feedback of stimulated ACTH responses occurs if a stimulus is applied in the minutes following an injection of the steroid. The magnitude of inhibition depends on the rate of rise of plasma GC and the effect lasts only as long as plasma GC concentrations are rising at a sufficient rate (Keller-Wood and Dallman, 1984). After a single injection or a constant infusion of GCs there is a period during which no inhibition of the ACTH response is observed if a stimulus is applied after steroid concentrations are no longer rising and before the delayed feedback effect has had sufficient time to develop. Delayed (intermediate and slow) feedback occurs if a stimulus is applied at least 60 minutes or more after a single injection of B or after B infusion. Maximal inhibition occurs when a stimulus is applied 2-4 hours after the steroid was administered, but the period during which some inhibition occurs is prolonged when high doses of steroid are administered, as delayed feedback is sensitive to dose.

Sites of Negative Feedback

The importance of maintaining GC secretion within tolerable limits requires efficient mechanisms for inhibiting stress-integrative PVN neurons (Herman and Cullinan, 1997). Injection of cortisol or DEX directly into the pituitary decreases B responses to stress (Rose and Nelson, 1956). Corticosteroids inhibit stimulated ACTH release from incubated pituitaries (Koch et al., 1974) and cultures of pituitary cells (Portanova and Sayers, 1974). Stimulation of ACTH secretion from a pituitary tumour cell line by AVP is inhibited by DEX or B 30 seconds prior to stimulation (Johnson et al., 1982). This suggests that the pituitary is sensitive to the fast feedback effects of B. Systemic administration of cortisol or DEX has been shown to decrease pituitary sensitivity to CRF preparations in animals with intact hypothalami (Arimura et al., 1969). Incubation of

disperses pituitary cells with DEX for 4 hours inhibits CRH-induced ACTH release, suggesting that the pituitary also shows delayed feedback responses to B (Portanova and Sayers, 1974).

GC injections into the PVN region downregulate CRH mRNA, decrease ACTH secretion and inhibit medial parvocellular PVN neurons, suggesting that GC negative feedback acts at the PVN neuron itself (Whitnall, 1993). The capacity for direct GC feedback is supported by evidence of expression of type II receptors (GR) in hypophysiotropic PVN neurons (Uht et al., 1988). However, feedback at the PVN cannot account for all aspects of HPA inhibition; inhibition of ACTH release occurs in the absence of a negative feedback signal (Jacobson et al., 1988) and total or anterior deafferentations of the PVN increase the expression of CRH and AVP mRNA, indicating that neuronal inhibitory pathways are required for maintenance of basal HPA tone (Herman et al., 1990).

Neuronal mediated inhibition of the HPA axis might emanate from several sources. The hippocampus displays the highest levels of GR binding, and GR and MR mRNA of any brain structure, suggesting a high degree of GC receptivity (Jacobson and Sapolsky, 1991). An inhibitory role of the hippocampus in HPA regulation is supported by lesion studies, which indicate that hippocampal damage potentiates stress-induced GC secretion in the rat and primate and increases the expression of CRH and AVP mRNA in parvocellular PVN neurons (Jacobson and Sapolsky, 1991; Sapolsky et al., 1991; Herman et al., 1995). Hippocampal stimulation results in a decrease of HPA activity in both rats and humans (Jacobson and Sapolsky, 1991). At present, however, the effects of the hippocampus on GC negative feedback remain controversial. In a study by Bradbury et al. (1993), there was no evidence to suggest that the removal of efferents from the hippocampus (fimbria-fornix lesions) decreased sensitivity of ACTH to B-mediated negative feedback inhibition. This was in contrast to studies that found that deafferentation produced by fornix lesions caused increased HPA activity (Wilson et al., 1980).

Other limbic system structures appear to convey some inhibition to the PVN. Prefrontal cortex (PFC) lesions (Diorio et al., 1993) result in enhanced HPA responses to acute stress. Implants of GCs into the prefrontal cortex block restraint-induced ACTH secretion as well (Diorio et al., 1993) and the PFC exhibits immediate early gene induction following acute stress (Cullinan et al., 1995). These stressor-inhibiting circuits are specific to the stressor being imposed. PFC lesions do affect responses to restraint but not to ether stress (Diorio et al., 1993). Hippocampal damage increases B responses to restraint but neither B nor ACTH responses to hypoxia (Bradbury et al., 1993).

Because the PVN receives input from hypothalamic circuits, lesion studies have indicated that local cell groups (BST, POA and hypothalamus) have the capacity to be involved in feedback inhibition. Ablations of the arcuate nucleus (ARC), medial preoptic area (MPOA), ventromedial nucleus (VMH) or suprachiasmatic nucleus (SCN) increase basal ACTH or B secretion and the magnitude and duration of the stress response (Viau and Meaney, 1991; Buijs et al., 1993; Larsen et al., 1994; Suemaru et al., 1995). All of these regions also contain populations of GABA-containing neurons and GABA is known to inhibit the release of ACTH and B *in vivo* (Makara and Stark, 1974). GABA also reduces CRH release from hypothalamic explants (Calogero et al., 1988). Finally, all of these cell groups show a rich expression of GR protein and mRNA, suggesting the potential for GCs to exert negative feedback action by way of hypothalamic cell groups (Herman, 1993). VMH lesions decrease the ability of low doses of B to inhibit baseline ACTH release and implants of B into the MPOA inhibit HPA responses to restraint and reduce AVP content in the median eminence (Viau and Meaney, 1996).

Basal HPA activity

There is a daily rhythm in adrenocortical system activity (Dallman et al., 1987). This circadian rhythm is thought to be generated by the suprachiasmatic nucleus of the hypothalamus (SCN) and regulation of the rhythm differs depending on the phase of the

light: dark cycle. Mean 24-hour basal plasma B levels in male rats average around 5 μg/dl. Low levels of B (0-5 μg/dl) are secreted throughout most of the light cycle, but 2-3 hours prior to the onset of dark, B levels begin to rise and peak just prior to, or at the onset of, the dark cycle (Dallman et al., 1987). B levels begin to decline thereafter such that by the end of the dark cycle, circulating levels of B are low. A number of groups have shown that there is an ultradian rhythm in both ACTH and cortisol in humans and rats (Windle et al., 1998; Veldhuis et al., 1989). Windle's group has shown that in the rat, circulating B concentrations display a dynamic ultradian pattern of release throughout the 24-hour cycle (Windle et al., 1998). The data in their studies suggested that pulse frequency did not change over the 24-hour period, which indicates that any pulse-initiating mechanisms that have influence over basal HPA activity have constant periodicity throughout the day. This suggests that the HPA axis is actively driven over the whole circadian cycle (Windle et al., 1998).

A rhythm in ACTH is also observed, but it is of much smaller amplitude than that of B. This rhythm is ultradian, since a number of peaks are seen throughout the 24 hour cycle and a distinct AM/PM difference is frequently not seen (Cascio et al., 1987). ACTH is known to occur in a pulsatile manner (Ixart et al., 1993; Boyle et al., 1997); the peptide shows a circhoral rhythm and variation over the 24-hour cycle (Carnes et al., 1989). Similar observations regarding the release of CRF have been made (Ixart et al., 1987; Lui et al., 1994). In general, the rhythm of hypothalamic immunoreactive CRH (ir) has two distinct peaks, one during the middle of the the AM phase and one following the onset of the PM, with a trough in CRH ir in between the two peaks that corresponds to the peak in plasma B levels (Moldow and Fischman, 1984).

Basal activity during the trough of the rhythm results mainly from constitutive secretory activity of the pituitary and adrenal components of the HPA axis without hypothalamic input; basal activity during the peak of the rhythm requires input from the

hypothalamus that is probably driven by signals from the SCN (Dallman et al., 1987). These conclusions are supported by a number of lines of evidence: 1) lesions of the basal hypothalamus (Kaneko et al., 1980), the PVN (Dallman et al., 1989) or the SCN (Cascio et al., 1987) do not decrease AM ACTH levels below their already low values but do prevent the normal PM rise in ACTH and B. 2) Passive immunization of rats with CRF antisera does not alter basal trough ACTH and B levels but does prevent the normal PM rise in both (Badgy et al., 1991). 3) Basal trough plasma B levels in intact rats are indistinguishable from those in ADXed rats, whereas basal peak plasma B is markedly elevated in intact but not ADX-ed rats (Dallman et al., 1987).

Circadian Activity

Circadian activity in the HPA axis is believed to be generated, in part, by the SCN (Dallman et al., 1987). Indirect inputs from the SCN to the PVN have been described (Berk and Finkelstein, 1981) and these may be partly responsible for the rhythmic change in basal activity which is characteristic of the HPA system (Cascio et al., 1987). The SCN lacks significant direct projections to the mpPVN (Buijs et al., 1993) but appears to innervate several hypothalamic cell groups that have been confirmed to provide afferents to the hypophysiotropic zone. These areas include the periventricular PVN and the dorsomedial hypothalamic nucleus (Buijs et al., 1993). This pathway is thought to be vasopressinergic: the high release of AVP from SCN terminals during the light period coincides precisely with low levels of circulating B at this time of day (Kalsbeek et al., 1996).

The PVN does not generate the rhythm independently of the SCN, since lesions of the SCN diminish (Abe et al., 1979) or abolish (Krieger et al., 1977) the normal rhythm in basal activity. The rhythm in plasma GCs and ACTH is entrained to the light:dark cycle. Constant light exposure disrupts circadian rhythmicity and can cause phase shifts in plasma B levels (Krieger and Hauser, 1978). A second major factor that determines basal activity in the HPA axis is feeding (Dallman et al., 1987). Rats consume more than two thirds of their

food in the PM phase of their cycle (Le Magnen, 1981). An association between adrenocortical activity and food intake was demonstrated in man in 1959 (see Krieger, 1979) and subsequent studies have shown that there are marked feeding-associated increases in plasma cortisol levels (Krieger, 1979). Johnson and Levine (1973) first reported that when rats were allowed only 30 minutes/day access to water, B levels were elevated just prior to the onset of the time of drinking and fell thereafter very rapidly. This effect obscured the normal circadian input from the light:dark cycle. Lesions of the SCN abolish the rhythm in B; this rhythm can be reestablished by food restriction (Dallman et al., 1987). Because both of these rhythms are normally in phase, then both most likely contribute to the normal amplitude of the circadian rhythm in basal activity of the system.

Feedback control of basal HPA activity

Basal HPA activity is subject to negative feedback effects of B during all phases of the cycle. Studies investigating negative feedback usually involve removal of B by ADX and replacement with exogenous levels of the steroid. ADX produces a rapid increase in the rate of ACTH secretion within 20-40 minutes (Dallman et al., 1972) and lasting 2 hours. During the first days following ADX, all components of the HPA axis show increased activity (Dallman et al., 1987). There is, however, from 2 to 6 and up to 48 hours following ADX, a decrease in ACTH levels in plasma that is associated with a decrease in pituitary ACTH content. The subsequent rise in pituitary ACTH content translates into increased plasma ACTH levels and this remains at a steady state of activity (Dallman et al., 1987).

In addition to increased corticotrope activity following ADX, 1-2 weeks following ADX corticotrope numbers have doubled (Rappay and Makara, 1981). ADX causes a rapid increase in bioactive CRF content in the median eminence at 2.5 minutes followed by a decrease in CRF content at 20 minutes after the removal of the adrenals (Keller-Wood and Dallman, 1984). Increases in mRNA in the parvocellular portion of the PVN of both CRH and AVP (Davis et al., 1986; Wolfson et all., 1985) are found following ADX. Further

evidence for the role of GCs in mediating negative feedback inhibition of basal HPA activity comes from work from Dallman et al. (1987). The rise in basal tough levels of ACTH after ADX is prevented by chronic replacement with very low amounts of B; under these conditions B also suppresses the ADX-induced synthesis of AVP.

Feedback inhibition of ACTH by B is subject to differential control in the AM versus the PM. Bradbury et al. (1991) showed that administration of antisera to CRF suppresses PM peaks in ACTH and B but has no effect on AM HPA function; administration of the antisera following ADX decreases both PM and AM levels of ACTH (Bradbury et al., 1991). ACTH concentrations during the trough of the rhythm are thus controlled by very low, constant circulating concentrations of B; however higher concentrations are required to inhibit peak ACTH secretion (Bradbury et al.,1991b). In fact, a PM increase in ACTH is still seen in ADX animals replaced with GCs (Akana et al., 1986) suggesting increased brain drive in the PM. Human studies suggest the same: treatment of people with metyrapone or the GC antagonist RU-486 stimulates ACTH secretion at peak but not trough times (Gaillard et al., 1984). These studies suggest that ACTH secretion is more sensitive to the inhibitory effects of GCs in the PM than in the AM, suggesting increased central drive in the PM. The occupation of GR and MR play an integral role in the regulation of basal HPA activity.

GR and MR-Basal HPA Function

The IC_{50} for the inhibition of ACTH in the morning, 0.7 nM of free B in plasma (Dallman et al., 1989), is very close to the K_d for the type I receptor and has been estimated to occupy 87% of the type I and 10% type II receptors (Veldhuis et al., 1982). In the evening the IC_{50} for the reduction of ACTH by free plasma B in ADX rats 5 days after surgery is 3.9 nM, five times that of the IC_{50} in the AM (Dallman et al., 1989). Doses of B at this level occupy approximately 95% of the type I receptors and 25% of the type II receptors (Veldhuis et al., 1982). In a study by Bradbury et al. (1994) the type I receptor

agonist, spironolactone, disinhibited plasma ACTH in the AM in ADX rats with either a low (2 μg B/dl plasma) or high (8 μg B/dl) negative feedback signal. In the evening, spironolactone increased ACTH in ADX rats with a higher B pellet dose, a treatment estimated to result in approximately 50% type II receptor occupation. Because it is clear that more B is required to reduce HPA activity in the evening, it has been proposed that there is a shift in control from the type I to type II receptor at this time of day (Bradbury et al., 1991). However the low efficacy of DEX alone in inhibiting plasma ACTH in the evening does not fully agree with this hypothesis (Bradbury et al., 1994). Only the presentation of DEX and B pellets together in the evening to ADX rats results in the inhibition of PM ACTH. These required PM levels would occupy GRs as well as Mrs.

PM levels of ACTH are thought to be maintained by the combined occupancy of MR and GR. In the study by Bradbury et al. (1994), DEX given to ADX rats reduced plasma ACTH only in the presence of very low concentrations of B, suggesting that for full potency, type II receptor occupation requires type I receptor occupation. Spironolactone administration to ADX-ed rats replaced with a high B signal, results in elevated ACTH throughout the entire circadian rhythm, suggesting the role of MR in regulating both Am and PM levels of ACTH. Ratka et al. (1989) found that, in the AM, i.c.v. administration of the MR agonist RU28318 produced an elevation of plasma B, but the GR antagonist RU38486 did not have an effect, suggesting that low levels of ACTH (and B) are maintained during the trough by B inhibition at MR sites.

The site(s) at which B acts to inhibit ADX-induced ACTH secretion appears to be the brain but where in the brain is still an open question. *In vitro studies* of pituitary and hypothalamus have shown direct inhibition by B of ACTH synthesis and secretion (Widmaier and Dallman, 1984) and CRF secretion (Nicholson et al., 1989). Dallman's group has performed a series of studies (reviewed in Bradbury et al., 1991) whether the same sites of feedback regulation are involved *in vivo*. Rats prepared with hypothalamic

and sham lesions (lesions prevented the endogenous secretion of CRF/AVP) were infused with CRF or vehicle. The rats were either ADX or sham-ADX. In the absence of either endogenous or exogenous CRF, ADX had no effect on any measures of ACTH production or secretion, demonstrating a complete lack of corticotroph autonomy in the response to ADX. In rats lesioned and given CRF, plasma and pituitary ACTH in the sham-ADX rats were lower than those in the ADX rats, providing clear evidence for inhibition by endogenous adrenal secretions at the pituitary. The conclusion here is that the hypersecretion of ACTH induced by ADX is a direct consequence of the normal action of B on the brain; under basal, trough conditions, the low B concentrations seen do not act at the pituitary to modulate the secretion of ACTH (Bradbury et al., 1991).

The hippocampus has been shown to be important for inhibitory tone over the HPA axis (DeKloet et al., 1998). Dorsal hippocampectomy or transection of the fornix elevates the basal HPA activity at the circadian trough in particular and CRH mRNA and AVP mRNA expression in the morning (Herman et al., 1989). In animals sampled throughout the day, i.c.v. administration of the MR antagonist elevated basal trough levels of plasma B (Ratka et al., 1989). In the afternoon phase, MR antagonists also elevated basal ACTH and B levels (Oitzl et al., 1995) as did a 10-fold lower dose injected bilaterally into the hippocampus (Van Haarst et al., 1997). A B implant into the dorsal hippocampus suppressed ADX-induced elevations in ACTH levels, while DEX implants were ineffective (Kovacs and Makara, 1988) which further supports the MR specificity of the response.

Stress-Induced HPA Activity

Organisms are constantly subjected to stimuli that can be interpreted as a stressor: Predators, climate changes, changes in the immediate environment, reproductive pressures, social interactions, illness or injury. In the laboratory setting a number of classical stressors have been used to stimulate the HPA axis. These include immobilization, restraint, predator, predator odours, social stress, cues associated with a previous stressor, insulin-induced

hypoglycemia, novelty, frustration, hemorrhage, handling, cold, footshock and ether. Interestingly, each stressor, by virtue of how its interpreted (psychologically, physically, emotionally, or a combination of any) will provoke a particular and unique response along the HPA axis. In general, however, the stress axis is controlled primarily by parvocellular neurons of the PVN. These neurons contain a number of neuropeptidergic adrenocorticotropin (ACTH) secretagogues, the most prominent of which are CRH and AVP (Antoni, 1986; Whitnall, 1993). Upon stimulation by stress or circadian drive, PVN neurons secrete these neuropeptides into the hypophysial portal system at the external lamina of the median eminence. CRH appears to be necessary for ACTH secretion and acts as the primary secretagogue, however factors such as AVP, which is co-stored and co-released with CRH in the parvocellular PVN, and oxytocin, which is probably derived from the magnocellular neighbor act in synergy with CRH at the corticotrope, potentiating pituitary release of ACTH. Once released, ACTH travels via systemic circulation to the adrenal cortex; here ACTH promotes both the biosynthesis and release of GCs.

Control of ACTH release

CRH, AVP, OXY and other neuropeptides have all been found in the portal circulation and have been shown to either produce or potentiate the release of ACTH from the anterior pituitary. Of particular importance is the notion that it is impossible to generalize about correlating the severity, duration and type of stressor and the resultant peptide alteration since the response to each particular stress is extremely unique. However there is an extensive amount of evidence implicating CRH as the main ACTH secretagogue and how CRH itself is modulated by other neuropeptides.

CRF-41 (Vale et al., 1981) and AVP have ACTH-releasing actions *in vivo* and their levels in the hypophysial portal blood bathing pro-opiomelanocortin (POMC) cells of the anterior pituitary reach concentrations which may alter ACTH secretion (Plotsky, 1991). Early studies using peptide antagonists and passive or active immunoneutralization indicated

that both of these factors play a role in the regulation of ACTH secretion under conditions where ACTH release is stimulated (Makara, 1993). Fairly large doses of antibodies to CRF-41 or AVP failed to counteract the ACTH-releasing effect of stressful stimuli completely, but the combination of CRF and AVP antibodies appeared to result in a blockade of ACTH secretion (Rivier and Vale, 1983). Van Oers et al (1988) showed however, that a large dose of monoclonal antibody to CRF-41 completely blocks the response to ether-induced stress in rats.

Physical stressors, including foot-shock, i.p. injection of hypertonic saline and nalaxone precipitated opiate withdrawal, as well as psychological stressors, including immobilization and swimming cause a significant increase in hypothalamic CRF mRNA (Lightman and Harbuz, 1993). Murakami's group found that stressor onset produced an increase in median eminence content of CRH but with no detectable levels of CRH in the PVN (Murakami et al., 1989). This increase in the median eminence is in fact transient, as CRH content begins to decline after the initial increase in response to ether; this decrease could reflect the release of CRH into the portal circulation and depletion of CRH stores in the median eminence.

AVP potentiates the effects of CRH on ACTH release but not induction of POMC (Whitnall, 1993). The primary site of AVP synthesis is the magnocellular neurons of the supraoptic and paraventricular nuclei, which project to the posterior pituitary. AVP is also synthesized in other regions of the hypothalamus, including the SCN and the CRF-containing cells of the medial parvicellular PVN (Whitnall, 1990). AVP acting on the adenohypophysis may derive from these parvicellular sources or in passing release into the median eminence by magnocellular projections or from short portal vessels connecting the anterior and posterior pituitary (Stout et al., 1995). AVP synthesis and release are not only increased in response to hypertonic or hypotensive stress but also in response to insulin-induced hypoglycemia (Burbach et al., 1984; Plotsky et al., 1985). In a study by Van

Dijken et al. (1993) a single session of repeated footshock produced long-term increase in AVP but not CRF in the median eminence. Whitnall (1993) has suggested that the CRH released into the portal circulation following stress is derived from the CRH+/AVP+ population of neurons in the median eminence. Exposure to acute stress resulted in depletion of secretory vesicles from CRH+/AVP+ terminals but not CRH+/AVP- terminals in the zona externa of the median eminence.

Oxytocin (OT) has been implicated in the response to a variety of stressors and in potentiating CRF-stimulated ACTH release (Antoni et al., 1983) in the rat but not in the primate (Gibbs, 1986). One minute of immobilization stress increases plasma OT and decreases hypothalamic OT concentrations (this stressor does not alter AVP concentrations). Increased OT release has also been demonstrated in response to ether stress but not to the stress of a novel environment (Gibbs, 1986).

ACTH response to stress

The response to stress by the HPA axis involves an increase in ACTH release, regardless of what time of day the stress is applied (Dallman et al., 1987). There is a strong relationship between increasing stimulus (or stressor) intensity and magnitude of ACTH secretion; once the stressor is terminated, however, ACTH levels begin to decline. If the stressor is of sufficient intensity, the decline in ACTH is delayed (Dallman et al., 1987).

A diurnal difference in ACTH and B responses to a variety of stressors has been described as larger in the AM phase of the cycle. By stressing ADX rats, Bradbury and colleagues (1991) have shown that the diurnal rhythm in stress responsiveness, in which ACTH responses in the AM are greater than in the PM, is seen in both SHAM and ADX rats. This suggests that this diurnal difference in HPA responsiveness to stress was not dependent on stress-induced increases in circulating levels of B. Finally, this difference was not due to a loss in sensitivity to secretagogues in the PM, since pituitary responsiveness to exogenously administered CRH and AVP was similar in the AM and PM. In addition to

this diurnal regulation in the ACTH response to stress, there are negative feedback mechanisms involved in terminating the response (see section on negative feedback). Fast and delayed feedback appear to act to inhibit stimulus-induced ACTH secretion only, whereas the slow feedback effects act to inhibit the expression of POMC mRNA and ACTH synthesis, resulting in inhibition of both basal and stimulated ACTH secretion (Keller-Wood and Dallman, 1984).

While the injection of exogenous B results in the inhibition of the B response to a subsequent stress, exposure to a stressor does not inhibit ACTH and B responses to a second stressor administered between 1 and 24 hours later (Dallman and Jones, 1973). In fact, exogenously administered B serves to inhibit further responses to stress, whereas prior stress seems to facilitate and inhibit further stress-induced responses and as a result, HPA activity in response to a second stressor is similar to the response to the first stressor. The questions that remain involve identifying what features of HPA activity serve negative feedback or facilitative roles and how these two processes jointly determine the magnitude of the response to stress. The origin of the negative feedback signal (from basal, prior stress-induced or ongoing HPA activity) has helped to explain why exogenous B but not stress-induced B elevations inhibit the response to a subsequent stressor.

Following ADX, basal and stress-induced ACTH responses are increased (Akana et al., 1988). When ADX rats are given B pellets that approximate normal basal levels, they still show increased ACTH responses to different stressors. Since ADX abolishes both the circadian and stress-induced increase in B, the absence of either B signal could account for the exaggerated ACTH response to stress. To further evaluate the relative roles of circadian and stress-induced increases in B in determining ACTH responses to stress, Jacobson et al. (1988) did the following study. Rats treated with cyanoketone (inhibits the conversion of inactive hydroxysteroids to active ketosteroids and thus inhibits B synthesis) (Felig et al., 1995) or vehicle on preceding days were exposed to an acute restraint stress either during

the daylight or dark hours. At all times the cyanoketone-treated rats exhibited at least a 65% reduction in the magnitude of the B response to this initial stress compared to the vehicle treated rats. Basal and stress-induced ACTH and B levels were measured in rats stressed during the previous 12-hour period and also in naive rats (not exposed to prior stress) at either the trough or peak of the diurnal rhythm. Compared to naive rats, prior stress had no effect on basal ACTH and B in the AM, in either vehicle or cyanoketone-treated rats. Prior stress elevated basal ACTH in the PM in both rats groups compared to their naive controls. Prior stress had no effect on the magnitude of the ACTH response to stress in the AM in vehicle treated rats, compared to their naive controls. In contrast cyanoketone-treated rats exposed to stress markedly hypersecreted ACTH, compared to their respective controls, in response to stress in the AM. The ACTH response to stress in naive cyanoketone-treated rats was not different from that in either previously stressed or naive-treated controls. These results provide direct evidence that prior stress induced facilitation of subsequent activity in the HPA axis (Dallman et al., 1992). Hypersecretion of ACTH occurs after stress applied in the AM in previously stressed rats that were unable to secrete a normal amount of B at the time of initial stress. The facilitatory action of initial stress on subsequent HPA activity enables animals to remain normally stress-responsive in the face of persistently, or previously elevated steroid feedback signal. The results of this study called into question the notion that stress-induced increases in B serve to inhibit the ACTH response to ongoing or continual stress and confirmed the role of circadian B as an adequate negative feedback signal in the control of ACTH responses to stress. These results confirmed how a single exposure to stress provides both the facilitatory and inhibitory signals for further stress-induced HPA responses, bringing these two signals into balance. Stress acts to facilitate subsequent responses in the adrenocortical system and this facilitation is balanced by the GC feedback signal related to basal or prior stress-induced HPA activity. GCs limits further responses of stress responsive systems so as to minimize harmful overexposure to

the catabolic GCs. However, facilitation allows for normal ACTH responses under conditions where repeated feedback signals should inhibit the response.

Negative Feedback Sites under Stressful Conditions-Role of MR and GR

Occupation of MR by endogenous ligand is 90% during morning trough levels of HPA activity (Reul and DeKloet, 1985). Similar levels of occupation (88-97%) were observed at the diurnal peak and after 1 hour of restraint stress respectively. Thus, MR are extensively filled with endogenous B under most circumstances, while the GR become more occupied concurrent with increasing plasma B concentrations due to stress of the diurnal rhythm (Reul and De Kloet, 1985). However, exclusive activation of GRs is insufficient to suppress the circadian peak and MR activation appears to be indispensable (Bradbury et al., 1994).

Occupancy of hippocampal GR seems to be particularly sensitive to changes in circulating B. Meaney et al. (1988) have shown that under basal B conditions about 15% of putative GR are occupied while following a 20 minute period of restraint stress, around 89% of the GR are occupied with a concomitant increase in GR translocation to the nucleus (from 15% to 57%). Another piece of evidence supporting the role of GR regulation of ACTH responses to stress is from a study by Akana et al. (1988). Higher levels of B were required to dampen stress-induced ACTH and PM ACTH levels in ADXed animals. The IC_{50} for ACTH inhibition in the PM is around the K_d for GR (2.5-5nM); this further supports the regulation of stress-induced ACTH by the binding of B to GR.

In vivo and in vitro experiments have demonstrated a role for the pituitary as a locus of negative feedback for stress-induced increases in ACTH. Corticosteroids inhibit stimulated (via administration of CRH, AVP. EPI, OXY) ACTH release from incubated perfused and superfused pituitaries (Rivier et al., 1982; Labrie et al., 1984; Link et al., 1993). The *in vivo* evidence is not as strong. Only high circulating levels of GCs can act at the pituitary to inhibit ADX-induced hypersecretion of ACTH (Levin et al., 1988). Finally

evidence from Spencer et al. (1990) suggests that the effects of B inhibition on ACTH release may be mediated by MR at the pituitary. Stress did not produce increased occupation of GR in the pituitary but did increase the occupation of MR. This suggests an insensitivity of the pituitary to high circulating levels of GR.

GCs are thought to act directly at the level of the PVN to inhibit ACTH and secretagogue release. Previous studies have shown that steroid implants (DEX or B) in the PVN region are able to inhibit the ACTH and B responses to neural stimuli (Feldman et al., 1992) and reverse ADX-induced upregulation of CRH and AVP (Kovacs et al., 1986; Sawchenko et al., 1987). These effects are presumably mediated via type II receptors as the PVN contains high amounts of GR immunoreactivity and mRNA (Herman et al., 1993; Fuxe et al., 1985). Other hypothalamic nuclei, such as the MPOA (< biblio >) and the arcuate (Magarinos et < biblio >) are also involved in stress response regulation. Lesions to the ventromedial nucleus of the hypothalamus (VMH) cause hyperactivity in the HPA system Dallman, 1984) which has been proposed to be mediated by occupancy of type I receptors (Suemaru et al., 1995). However, other evidence has shown that extrahypothalamic afferents to the PVN are required for maintenance of CRH and AVP expression (Herman et al., 1990) and local application of GCs or antagonists into extrahypothalamic sites can modulate HPA activity (Kovacs and Makra, 1988). Thus it is likely that other GC sensitive regions within the CNS are capable of modulating HPA axis activity via neuronal projections to the PVN.

One brain region frequently implicating the feedback effects of GCs is the hippocampus. The hippocampus contains very high levels of GR and MR and has been suggested to play a role based on hippocampal lesion and B implant data (Magarinos et al., 1987; Sapolsky et al., 1984, 1990; Plotsky et al., 1987). Dorsal or complete hippocampectomy augments plasma B in response to surgery stress (Feldman and Conforti, 1980) and destruction of more than 50% of the neurons in the hippocampus enhances

restraint-induced increases in plasma B (Sapolsky et al., 1984). The hippocampus may also affect the termination of HPA responses to stress (Sapolsky et al., 1984); rats with hippocampal lesions have sustained elevations in B one hour following the termination of restraint stress. Available evidence suggests that most known ACTH secretagogues are influenced by hippocampal input. Fornix transection reduces the sensitivity of CRF, AVP and OT to B feedback in a seretagogue specific manner and as the fornix carries efferent projections from the hippocampus to the septum, these data are further evidence for a hippocampal role in negative feedback regulation.

The hippocampus contains high concentrations of MR and GR and a number of groups have examined the role of MR and GR in the hippocampus in negative feedback (Jacobson and Sapolsky, 1991). Sapolsky et al. (1990) demonstrated that occupancy of hippocampal GR is negatively correlated with portal levels of CRH and AVP but that CRH was related to the extent of both MR and GR occupancy in the hippocampus. Bradbury and Dallman (1989) showed consistent data: in ADX rats replaced with constant levels of B and treated with the MR antagonist spironolactone showed elevations in AM and PM ACTH, suggesting the involvement in both types of receptors in the inhibition of circadian peak of ACTH secretion.

While most of the lesion data show an elevation in adrenocortical activity, there are a number of reports that show either no effect of fimbria-fornix lesions (Bradbury et al., 1993) or an inhibition of unstressed and stressed B secretion following hippocampal or fornix lesions. Some of the contradictory results have been explained by the variability in recovery time between lesion and experiment. Rats with fornix lesions were found to have no circadian B rhythm 1 week after surgery but had a normal circadian peak and trough by 3 weeks post-surgery (Lengvari and Halasz, 1973). Bradbury et al. (1993) propose that fornix lesions may cause a shift in the site of feedback from the hippocampus to another area with B receptors.

The medial region of the rat prefrontal cortex contains both MR and GR and these sites share the binding properties of MR and GR described elsewhere in the brain (Reul and De Kloet, 1985). Lesions of the cingulate gyrus of medial prefrontal cortex (MpFC) were associated with significantly increased plasma ACTH and B in response to stress (Diorio et al., 1993). Further, B implants into this same region significantly reduced plasma ACTH and B responses to stress suggesting an inhibitory effect of GCs on stress-induced HPA activity. Implants of DEX or B into the lateral septum (Dallman and Yates, 1968) reduce stress levels of B and lesions of the central nucleus of the amygdala attenuate HPA responses to restraint (Beaulieu et al., 1987).

Chronic Stress

There is substantial evidence that chronic stress contributes as a significant risk factor to the expression of disease. These include diabetes (Surwit et al., 1991; Cox and Gonder-Frederick, 1991), gastrointestinal disorders, including ulcers (Weiner, 1992), heart disease (Jacobs et al., 1992), cancer, viral infections and autoimmunity (Cohen et al., 1991; Spiegel et al., 1989), neurological disorders including depression, Alzheimer's and memory loss (Arborelius et al., 1999; Deshmukh and Deshmukh, 1990; Lupien et al., 1998) and hypertension (Lawler and Cox, 1985). Chronic changes in HPA activity have been correlated with the onset, progression and relapse of these disorders.

Chronic stress is a difficult concept to define because there are a number of models that have been developed to simulate chronic activation of the HPA axis. The first paradigm is the chronic, intermittent stress paradigm, where animals are exposed to the same stressor on a daily basis for a certain period of time during the day and for any number of days. For example a 3 hour period of restraint given daily for three consecutive weeks would be an example of this kind of paradigm. Another intermittent chronic stress paradigm is the same as the one previously described with the exception that the stressor changes from day to day. This has been termed "variable unpredictable stressor" and

differs from the intermittent chronic stress. Here, for example, on day one the animal would receive a 3 hour period of cold, on day two, a three hour period of cat odor, on day three, a one hour swim stress, etc... for a period of three weeks. The second paradigm is a continuous chronic stress, which is exposure to a stressor for 24 hours per day for a number of days. 24 hours per day of cold for three weeks would be an example of this kind of stress. The final chronic stress is one where a disease state is present, such as streptozotocin (STZ)-induced diabetes or a chronic immunologically mediated inflammation such as adjuvant arthritis. The kinds of paradigms used in the rodent studies to be described are either chronic intermittent or chronic intermittent with the unpredictable stressor component and, by virtue of the disease state caused by dietary manipulations, we have used the chronic stress model where a disease state is present.

Chronic Stress with Disease State

Common responses of the adrenocortical system to both repeated punctuate and sustained stressors include: increased pituitary corticotrope metabolic activity (Pollard et al., 1976), increased adrenal weight and increased AM plasma B levels compared to control rats (De Nicola et al., 1977; Vernikos et al., 1982; Gibson et al., 1985). In streptozotocin (STZ)-induced diabetes, rats are injected with STZ in the tail vein and usually studied 5 days following injection. Rats exposed to STZ-induced diabetes have been shown to exhibit exaggerated plasma B responses to novel, acute, stimuli (Daniels-Severs et al., 1973; De Nicola et al., 1977) and decreased sensitivity to GC negative feedback compared to control rats (Young et al., 1990). In a study by Scribner et al. (1991), single samples of basal plasma ACTH and B did not reveal significant differences between vehicle and STZ-treated rats. Nonetheless, STZ-treated rats exhibited consistent increases in adrenal weight, decreases in thymus weight as well as elevated urinary B output. STZ-diabetic rats hyperesponded to the acute stress of histamine injection but did not exhibit altered pituitary sensitivity to CRF and/or AVP; they also exhibited normal adrenal responsiveness to

ACTH. Finally, the B response to acute stress was not as effectively inhibited by DEX as in the vehicle-treated controls. Thus, the chronic stress of STZ-induced diabetes causes both tonic hyperactivity of the adrenocortical system. which is possibly dampened but not abolished by the chronically elevated but not relatively ineffective feedback signals, and facilitates the activity of central neural components of the adrenocortical system (Scribner et al., 1991).

In the case of adjuvant or mycobacterial arthritis, the inflammation becomes apparent between 11 and 16 days after injection and reaches a peak 21 days, after which the acute phase subsides (Harbuz and Lightman., 1992). The development of the arthritis is assessed by paw volume, i.e. the number of swollen joints in the hind paws. In terms of HPA activity, a number of studies have reported increased circulating levels of B, increased adrenal weight and increased POMC mRNA and ACTH content in the anterior pituitary (Harbuz and Lightman., 1992). When the first signs of arthritis are evident there is a coincident fall in CRF mRNA in the PVN, a fall in CRF release into hypophysial circulation and an increased release of AVP into portal blood. Thus in chronic disease states such as adjuvant arthritis and STZ-diabetes, mean AM levels of B are elevated (resulting in a loss of diurnal rhythm), adrenal weight is increased as are POMC mRNA and ACTH content in the anterior pituitary.

Chronic, Intermittent Stress

Similar Daily Stressor paradigm

Adrenal size and plasma and adrenal B levels increase initially in response to chronic stress, but subsequently return to baseline levels in spite of continued exposure to the stimulus (Seyle, 1946). Stressors, including repeated footshock (Kant et al., 1985; Pitman et al., 1990), repeated restraint (Hashimoto et al., 1988), ethanol stress (Spencer and McEwen, 1990) and intermittent noise Amario et al., 1986) cause plasma B elevations for up to one week into the stressor, but these levels subsequently fall back to control levels.

However, Vernikos et al. (1982) found that exposure to 2 weeks caused increased adrenal weight and decreased thymus weight and Ottenweller et al. (1994) found that AM B levels were consistently elevated in response to restraint-shock sessions, but that PM B levels were similar in stressed and control rats. Thus increased HPA activity is not consistently seen during or following chronic intermittent stress paradigms and if alterations in basal B are seen, it is usually confined to the circadian trough.

ACTH levels do not remain elevated in chronic stress paradigms (Daniels-Severs et al., 1973; Hashimoto et al., 1988) although in one chronic restraint stress paradigm, while ACTH levels fell with repeated exposure, elevated plasma B concentrations persisted over 16 days (Hauger et al., 1990). Amario et al. (1986) showed that animals exposed to chronic noise stress for 22 days had lower B responses to the 22nd noise compared to animals exposed to the noise for the first time. In response to chronic cold, Daniels-Severs et al. (1973) reported that increased B for one week, falling to levels below controls after 8 weeks. Vernikos et al. (1982) reported that circulating levels of both ACTH and B were greater compared to controls at all times over two weeks. Bhatnagar et al. (1995a) found that in rats exposed to chronic intermittent cold, basal plasma B and ACTH, CBG and EPI were similar in stressed and control animals, while catecholamine enzyme activity was higher in the chronically stressed rats. Adaptation to the same (or "homotypic") stressor is an efficient response to a threat which becomes predictable and familiar, although, as outlined above, adaptation to chronic stress is not uniform across all paradigms.

While, for the most part, responses to the same repeated stressor habituate, the presentation of a novel (or "heterotypic") causes a hyper-responsiveness of the HPA axis (Vernikos et al., 1982; Young et al., 1990; Ottenweller et al., 1989; Scribner et al., 1991; Opstad, 1991; Bhatnagar et al., 1995) and a decreased sensitivity to exogenously administered GC-feedback signals (Vernikos et al., 1982; Scribner et al., 1991). This facilitation to the novel stressor enables the animal to remain normally stress responsive to

an unencountered and unpredictable stress. Facilitation does not occur in all chronic stress paradigms. When animals are chronically injected with ethanol (Spencer and McEwen, 1990) or intermittently restrained (Kant et al., 1985) exposure to the heterotypic stress does not result in facilitative HPA responses to the novel stressor.

Variable, Unpredictable Chronic Stress

The chronic sequential exposure of rats or mice to a variety of mild stressors as used in the chronic mild (unpredictable) stress (CMS) paradigm reportedly produced performance deficits in behavioral paradigms that measure responsiveness to rewards (Azpiroz et al., 1999). As stress is said to be implicated in the etiology of depression (Brown and Harris, 1988), the CMS paradigm appears to provide a relatively realistic animal model of the decreased response to rewards (anhedonia) that characterize depression (Fawcett et al., 1983), facilitating the study of concomitant changes in brain and immune function. The stress regimen consists of variable stressors, such as water deprivation, acute cold, acute restraint, acute swim, continuous illumination, cage tilt, intermittent sound, predator odour and acute isolation. Stressors get programmed throughout the time period prescribed) and are changed everyday so that animals are unable to predict which stress they are going to receive on any given day. In terms of HPA axis activity, results from very few studies have been inconsistent. Azpiroz et al. (1999) found that after 4 weeks of the CMS paradigm, B levels were similar to unstressed controls, either following 4 or 7 weeks of stress. Ayensu et al. (1995) reported higher B levels in rats after 4 weeks of CMS. It has been suggested that whether adaptation occurs is dependent on the intensity of the stress (Pitman et al., 1988); since studies using the CMS paradigm apply a variety of stressors of differing intensities, it is difficult to compare the results of these studies.

Changes in Central Components of the HPA Axis

Chronic stress paradigms with enhanced ACTH responses to a novel stimulus are associated with different degrees of stimulation of CRH expression (Bartanusz et al., 1993;

Imaki et al., 1991; Kiss and Aguilera, 1993; Lightman and Harbuz, 1993). Moreover, while repeated footshock or i.p. hypertonic saline injections do not produce a desensitization of ACTH are associated with sustained increases in CRH mRNA in the PVN (Imaki et al., 1991; Kiss and Aguilera, 1993; Lightman and Harbuz, 1993), the desensitization of the ACTH responses to the primary stress observed during chronic immobilization or cold exposure is associated with a more transient elevation in CRH mRNA, detectable only after the repeated stress (Bartanusz et al., 1993). In adjuvant arthritis models of chronic stress, there is a consistent fall in CRF mRNA in the PVN that is coincident with the first signs of arthritis, in addition to a corresponding decrease in CRF-41 in portal blood (Harbuz and Lightman, 1992). AVP release into portal blood was increased, suggesting that in the presence of permissive levels of CRF, AVP may activate the HPA axis (Harbuz et al., 1992). Finally, Hauger et al. (1990) found that chronic restraint results in a loss of anterior pituitary CRF receptors.

Young et al., (1990) found a lack of fast feedback inhibition of corticotroph responses in rats exposed to an intense chronic intermittent swim stress and suggested that lower steroid receptor concentrations might be involved in this diminished fast feedback. In fact, studies in the rat have shown that the levels of GR in the hippocampus and anterior pituitary are decreased following chronic stress (Sheppard et al., 1990). Sapolsky et al. (1990) found decreased receptor densities in hippocampus and amygdala in animals exposed to chronic intermittent stressors for 3 weeks and Spencer and McEwen (1990) found evidence for changes in MR densities in both hippocampus and forebrain.

Chronic stress in humans

Chronic stress in humans has been investigated under different paradigms: there are physical stressors such as exertion, heat, cold trauma, infection, and inflammation, and psychological stressors such as fear and anxiety, social stress, humiliation, and dissapointment. Individuals use a diverse array of coping mechanisms and their

environmental and genetic backgrounds influence both the response and manner with which they deal with stress. In addition, the perception of the stressor is critical and can profoundly affect both the actual response of the individual to stress and the outcome from the stressor itself. Finally, the effects of acute stress will often synergize with chronic stress (myocardial infarctions, for example) and potentially exacerbate and hasten the onset and progression of disease. Thus in reviewing chronic stress in humans it is imperative to keep all of these factors as contributing to the appearance of pathology.

Basal HPA function in Humans

Before describing the effects of stressors on HPA activity in humans, a summary of basal HPA axis function is warranted. Despite the extensive research exploring HPA axis production of cortisol, less is known about the diurnal cycle of cortisol. Cortisol and ACTH are secreted in a pulsatile fashion, having both ultradian and circadian rhythms. ACTH and F secretion occur in 16-19 rapid bursts of secretion per day. Furthermore, it has been shown that the secretory pulses of F and ACTH are significantly concordant, with cortisol pulses lagging very each ACTH secretory burst by about 10 minutes (Smyth et al., 1997). In adults who are on a typical day/night cycle, peak levels of basal F are produced during the last hours of night-time sleep (Anders, 1982). This results in high early morning levels that help sustain energy for action and stimulate the appetite. Recent evidence suggests that free F levels increase by 50-75% within the first 30 minutes after awakening in both men and women (Pruessner et al., 1997), suggesting that waking up in the morning is a potent stimulus for the HPA axis. Women show larger increases in early morning F when compared to men (Pruessner et al., 1997). In a study by Van Cauter et al. (1996), that as young adults, overall plasma F levels are lower in women than in men; however, in men the profiles of F secretion were characterized by higher and more prolonged early morning elevations. Interestingly, interindividual variability in the magnitude of the morning elevation was larger in men than in women (Van Cauter et al., 1996). Born et al. (1995)

found that men and women had comparable F concentrations throughout the sampling period employed (16:00-19:00) but basal ACTH concentrations were higher in men than in women. In general, early morning peak levels decline sharply during the first few hours after waking and more gradually thereafter with F production increasing again during sleeping hours (Felig et al., 1995; Weitzman et al., 1971). Basal levels of F follow a circadian rhythm in healthy adults that is modulated by the timing of normal daily activities such as napping and meals (de Kloet, 1991). In a study by Smyth et al. (1997) individual differences in the diurnal cycle of cortisol were examined. They found three distinct subgroups that could be differentiated based on their rhythm in F: those with a typical diurnal (declining across the day) rhythm in F, those with no diurnal pattern at all and those who showed different diurnal patterns on each day they were tested. The diurnal cycle was not related to any demographic or psychological variable that that was examined; clearly this study emphasizes the need for additional investigations into the mechanisms for the observed individual differences in F production.

Acute and Chronic Stress-Induced Changes in the Human HPA axis

Acute stressors can potently activate the HPA axis in humans. A study by Amario et al. (1996) examined the F, glucose and prolactin (PRL) response to an examination stress. Their results indicated that anxiety-provoking situations, such as an immediate anticipation of an examination increased plasma F, PRL and glucose. Prolactin not only responded to stress but its response was significantly different between two stressful situations differing in the amount of anxiety they provoked. By contrast, plasma glucose responses, while increased in response to the stressor, did not reflect the differences in the anxiety caused by the various situations. There are, however, discrepancies in the literature on the reliability of pituitary-adrenal and PRL responses to acute stress in humans. Positive F and PRL responses have been observed in various psychologically stressful situations (Corenblum and Taylor, 1981; Kemmer et al., 1986; McCann et al., 1993; Meyerhoff et al., 1988; Stahl

and Dorner, 1982). Most negative reports could be partially explained by the temporal relationship between blood/saliva sampling and the stressful experience. First, Johansson et al. (1983) observed a significant increase in PRL immediately before an examination, with a rapid return to control levels. Second, other authors did not find any change in F, PRL or GH during the week of examination (Allen et al., 1985; Malarkey et al., 1991) or in F or PRL after 3 hours of examination (Semple et al., 1988); in contrast significant increases in F and PRL were found 15-20 minutes after the students had taken the exam (Allen et al., 1985). Third, whereas no endocrine changes have been observed in parachutists on the days or hours preceding the jump (Noel et al., 1976), significant increases in anterior pituitary hormones and F have been demonstrated immediately after the jump (Noel et al., 1976; Schedlowski et al., 1991). Accordingly salivary F concentrations increases after a wide range of anxiety-provoking situations, including examinations (Stahl and Dorner, 1982). When the stress is imposed may also be critical: a rise in F in response to mental stress has been observed during the time of low circulating F, but not when its levels are high (Holl et al., 1984).

There are also effects of gender that are involved in the response to an acute stress. In the study by Born et al. (1995), there were significant gender effects on the cortisol response to an injection of CRH. In women the increase in cortisol concentration after the injection was prolonged and the recovery of basal concentrations appeared to be delayed (Born et al., 1995). In fact, sex differences were even more pronounced after the combined stimulation with CRH/AVP than with CRH alone.

A number of studies have explored natural chronic stressors and cortisol secretion: repeated parachute jumps, social stress, work stress, death and grieving, combat exposure and PTSD, unemployment, accident and trauma, and repeated stressors such as repeated military training, and repeated psychological stress. Deinzer et al. (1997) found that when individuals did 3 consecutive parachute jumps, adrenocortical responses in response to the

jumps were significantly higher than many other common stressors. The mean cortisol increase and peak responses were similar in the first two jumps and were significantly higher than the F response to the third jump. This is in agreement with numerous other studies that have reported unchanged F responses to stressors repeated once (Dallman and Jones, 1973; DeSouza and Van Loon, 1982; Wittersheim et al., 1985). Kirschbaum et al. (1995) did a study that examined the response to repeated psychological stressors. Male subjects were each exposed to five times to the same brief psychosocial stressor (public speaking and mental arithmetic in front of an audience) with one stress session per day. Cortisol levels were significantly elevated on each of the five days of stressor testing. The mean F response decreased from day 1 to day 2; however no further attenuation could be observed on the remaining days.

Job/occupational stress is another chronic stress shown to cause alterations in HPA activity. Brantley et al. (1988) found elevated urinary cortisol levels in the afternoon on days with a high number of daily stressors compared to days with a low number of daily stressors. Caplan et al. (1979) found that greater work demands were associated with lower F in the morning but not in the afternoon. These individuals also did not have the expected decrease in F from morning to afternoon. Elevated F levels were associated with the assessment of "bad" versus "good" days at work (Lundberg et al., 1989). In contrast Cummins and Gervitz (1993) did not find any relationship between the number of undesirable events of a day and evening urinary or salivary F. Lindquist et al. (1995) found no direct associations between perceived stress and blood pressure after allowing for lifestyle factors in male and female office workers; this was also confirmed in a later study by Lindquist et al. (1997) which elaborated on the role of lifestyle factors and coping mechanisms in blood pressure responses to both perceived and actual occupational stressors. Men reported higher levels of alcohol consumption and unhealthy eating and lower levels of healthy eating when compared to women's' strategies used to cope with stress. This brings up an important issue about individual variation in stress responding.

Kirschbaum et al. (1995) describe subjects as being "low" and "high" responders (both in terms of basal and stress-induced HPA function). When subjects are exposed to a repeated stressor over 5 days, low responders show elevations in F only on the first day of stressor testing. In contrast, "high responders" display large increases in F in response to the stressor on each day of testing and do not show any decrement on day 2 of stressor testing to the same stressor.

In addition to F levels and blood pressure in response to occupational stressors, several studies have documented an increase in cholesterol levels due to occupational stress, natural disasters, job loss and academic examinations (Friedman et al., 1958; Trevisan et al., 1986; Gore, 1978; Kasl et al., 1968; Grundy et al., 1959; O'Donnell et al., 1987; Francis, 1979). More recently, Niaura et al. (1991) examined the lipid and lipoprotein responses to occupational (tax accountants) and academic (medical students undergoing examinations) stress. The occupational stress of tax season and the anticipation of final examinations did not affect lipid, lipoprotein and apolipoprotein levels; the authors explain that perhaps only large magnitude stressors that are longer term in nature can provoke changes in lipid levels.

While occupational and academic stressors can provoke changes in HPA activity, blood pressure and lipid levels, the chronic stressor of unemployment has been examined in the same context. Ockenfels et al. (1995) have shown that individuals who has been unemployed, on average for 12.5 months, had higher morning and lower evening urinary F levels when compared to employed subjects. They also examined the salivary cortisol reactivity to acute daily stressors and found no difference between employed and unemployed subjects. Arnetz et al. (1991) found that unemployed men and women had higher serum F levels compared to employed subjects. F levels were elevated during the anticipatory phase of unemployment, returned towards base-line levels during the following 6 months and increased again around the 12-month mark before declining again.

Social stress has been used as a chronic stressor as well. Sapolsky (1992) has reviewed the literature on chronic social stress and in general, elevated basal GC levels are observed in subordinate animals of many species, such as baboons, monkeys, and rats, mice and tree shrews. This overactivation of HPA activity has been linked to cortcosteroid receptor downregulation and blunting of feedback efficacy, which is what is usually seen in clinical depression. In a clever experiment by Hellhammer et al. (1997), army recruits were examined at the beginning of boot camp training. This allowed for an examination of the effects of chronic stress and social rank on adrenocortical activity. First, baseline F levels increased over the first five weeks, suggesting that recruits were under constant stress. This increase in F was independent of any individual's rank within the social hierarchy. Under a psychological stress (public speaking) salivary F levels highly increased in socially dominant subjects, while only a modest elevation occurred in subordinate men. A similar phenomenon has been described in rodents: in the Visible Burrow System (Blanchard and Blanchard, 1990), groups of rats are housed together for two weeks and as a result, a dominance hierarchy develops. For all rats housed in the VBS, plasma B levels are significantly elevated when compared to control rats housed in standard laboratory cages. However, a subgroup of subordinate rats show an impairment in their ability to produce the characteristic rise in plasma B when presented with a novel stressor (Blanchard et al., 1993). The mechanism for these rats' blunted B response to the novel, heterotypic stress are reduced levels of CRF mRNA in the PVN, resulting from enhanced inhibitory input to PVN neurons (via increased GC feedback). In human, this lack of response in subordinate men may result from a lack of involvement and engagement from the stress (Hellhammer et al., 1997). Friedman et al. (1963) observed an attenuated 17-OHCS reactivity to an acute stressor (medication error) in parents of children going thorough a terminal illness. Soldiers in Vietnam also showed suppression of 17-OHCS levels during anticipation of an acute stressful period (an impending enemy attack) (Bourne et al., 1968) and post-

traumatic-stress-disorder (PTSD) patients show blunted F responses to CRH injections (Smith et al, 1989).

PTSD is an interesting chronic stressor. Most studies examining PTSD have demonstrated significant differences in HPA axis parameters in PTSD sufferers compared to normal controls. Many of the studies performed indicate that individuals with PTSD show evidence of significantly lower mean 24-hour F levels (Mason et al., 1986; Yehudah et al., 1990) when compared to other psychiatric conditions and normal controls. In contrast, another group has found that urinary F excretion was significantly higher in combat veterans with PTSD compared with those veterans without PTSD (Pittman and Orr, 1990). When subjects are sampled for plasma B every 30 minutes for 24 hours, basal plasma F was significantly lower, primarily in the late evening and early morning hours of the PTSD group. Low cortisol in PTSD is consistent with the finding of significantly larger numbers of GR in lymphocytes (Yehuda et al., 1993). In terms of the response to challenge, PTSD is commonly associated with an enhanced suppression of cortisol to low doses of DEX and a blunted ACTH response to CRF challenge (Yehuda et al., 1995). A recent investigation by Lemieux and Coe (1995) reported that women who had suffered childhood sexual abuse and currently suffered from PTSD showed higher levels of NE, EPI, dopamine and F and showed a tendency towards obesity. In terms of central changes in PTSD, Bremmer et al. (1995) found that patients with PTSD had a smaller right hippocampal volume when measured by MRI and this decrease in volume was associated with functional deficits in verbal memory.

A link between depression and stress is suggested by the association of stressful life events with the onset of depression (Paykel, 1979) and from observations that changes occurring in response to chronic stress including increased anxiety, decreased appetite and libido and increased HPA and sympathetic activity are changes that also occur in depression (Gold et al., 1986). Stressful events often precede the onset of depression and stress has

been associated with the severity of the illness (Hammen et al., 1992). Stressful life events in childhood have been shown to predispose an individual for the development of mood and anxiety disorders later in life (Arborelius et al., 1999). These early life events may increase an individual's vulnerability to stressors later in life by inflicting biological damage and thus, predispose an individual towards affective pathology.

A compelling number of studies have found several measures that indicate hyperactivity of the HPA axis in depressed patients (Plotsky et al., 1995). Board et al. (1956) were the first to find elevations in plasma F concentrations in a majority of patients with major depressive disorder. This result has been replicated many times. Additionally, depressed patients show lower DEX suppression: a single dose of DEX (dexamethasone suppression test or DST) suppresses plasma ACTH, b-endorphin and F concentrations to a lesser extent and/or for a shorter amount of time in depressed patients. After iv administration of CRF, depressed patients exhibit a blunted ACTH but normal F response compared to healthy controls (Gold et al., 1986). Further, elevated CSF CRH concentrations have been found in depressed patients (Nemeroff et al., 1984) and decreased CRF receptor binding sites have been found in the prefrontal cortex of suicide victims (Nemeroff et al., 1988). Elevated CRF concentrations and CRF mRNA have been found in the hypothalamic PVN of depressed patients (Raadsheer et al., 1995).

The link between early life stress and the development of depression later in life has been well documented in animals (non-human primates, rats) but is less well documented in humans. However, a number of studies suggest that stressful childhood events have an impact on the development of depression and anxiety. For example, loss of a parent in childhood was found to increase the risk for major depression and generalized anxiety disorder in a retrospective twin study (Kendler et al., 1992). Women who reported having been physically and/or sexually abused as children had higher scores for both depression

and anxiety, lower scores of self-esteem and were more likely to have attempted suicide than those not abused as children (McCauley et al., 1997).

Other physiological functions of GCs

Aside from their numerous effects on intermediary metabolism (described later), glucocorticoids are ubiquitous as physiological regulators and play a role in differentiation and development. They are essential for survival and for the adaptive response to stress.

GCs and immunologic and inflammatory responses.

GCs in excess suppress immunologic and inflammatory responses (Baxter, 1979; Harbuz and Lightman, 1992; Schobitz et al., 1994). Preincubation of endothelial cells (which direct the traffic of leukocytes into inflamed and infected areas) with GCs markedly inhibits the induction of leukocyte movement, which in turn reduced leukocyte binding to endothelial cells (Cronstein et al., 1992). GC administration is also followed by a decline in the number of circulating lymphocytes (Claman, 1972). Both T and B-lymphocytes are depleted. Endogenous GCs induce apoptosis of thymocytes, which may prevent the appearance of autoaggressive T cells after immune activation (Schobitz et al., 1994). Pharmacological doses of dexamethasone decrease natural killer cell activity (Holbrook et al., 1983), and block the proliferation of mitogen-stimulated peripheral blood lymphocytes (Nowell, 1961). Because cytokines play a key role in the immune response to injury, the control of cytokine synthesis appears to be a hallmark of GC action. GCs inhibit the production of numerous cytokines (IL1, IL2, IL6, TNF, prostaglandins, etc...). GCs also inhibit the induction of nitric oxide (NO) synthase in a number of tissues, thereby attenuating vasodilation in inflamed tissues. GCs at low concentrations are required, however, for an optimal biological response. In fact, an analysis of the relationship between immunological effects and the GC concentration reveals 2 different dose response curves. First there are effects with the maximal immune response occurring in the absence of

endogenous GCs (adrenalectomized rats). The higher the GC concentration, the more the response is suppressed.

Cardiovascular system, bone and calcium metabolism, gastrointestinal tract and adrenal medulla

In GC-deficient states there is hypotension with decreased responsiveness to pressor stimuli and decreased cardiac output. In GC excess states there is hypertension. GC excess can ultimately lead to osteoporosis by decreasing bone accretion and increase bone resorption, resulting in an overall loss of bone mineral (Felig et al., 1995). GCs at high doses inhibit DNA synthesis in gastric but not jejunal mucosa and increase the incidence of gastric ulceration (Felig et al., 1995). Finally, the adrenal medulla receives adrenocortical venous effluent and thus is exposed to much higher cortisol concentrations than are other tissues. GCs affect adrenal chrommafin cell characteristics and the catecholamine biosynthetic pathway. They regulate phenylethanolamine (PNMT), tyrosine hydroxylase (TH) and dopamine B-hydroxylase activities.

Growth, development and reproductive function

GCs in excess inhibit linear growth and also inhibit skeletal maturation (Baxter, 1978). GCs also inhibit growth and cell division in a number of individual tissues. There are, however, variations in sensitivity: in a growing rat, liver, heart, muscle and kidney are more sensitive than gastric and jejunal mucosa, spleen, brain and testis (Loeb, 1976). In vitro, GCs can either stimulate or inhibit cell division; stimulation may occur by a steroid-induced augmentation of the actions of growth factors such as fibroblast growth factor and IGF-1 (Conover et al., 1985). Conversely, inhibition could be due to actions of the steroid blocking growth factor production and/or action or due to other inhibitory actions of GCs on the cell. GCs inhibit growth hormone, but this action probably does not explain the growth-inhibiting effects of the steroid, since they are not overcome by the administration of GH. GCs in general, tend to affect the timing and rate of cellular differentiation, but not the

sequence of developmental events (Felig et al., 1995) and GC sensitivity during development varies tremendously. GCs generally inhibit reproductive function by inhibiting LH release and in turn, suppressing testosterone production. In females, GCs also suppress basal and gonadotropin releasing hormone-stimulated LH levels, plasma estrogen and progestin concentrations, ovulation and the onset of puberty (Novotny et al., 1986).

Synergistic and antagonistic interrelations between GCs and other hormones.

Insulin resistance and the relationship to GCs.

Himsworth (1936) was one of the first to remark that a large number of his patients with diabetes were "insulin insensitive"; based on this he suggested that his patients be divided into 2 categories- insulin sensitive and insulin insensitive. These distinctions later became known as insulin-dependent (IDM) and non-insulin dependent diabetes (NIDDM). One major hallmark of NIDDM is some degree of insulin resistance, which is an impairment of insulin-mediated uptake of glucose by tissues, predominantly muscle (Reaven, 1988; 1995). Kahn (1978) defines insulin resistance as existing whenever normal concentrations of hormone produce a less than normal biological response. Three main mechanisms have been proposed to contribute to insulin resistance: alteration of insulin-stimulated glucose transport, inhibition of intracellular glucose metabolism or impaired insulin-induced increase in muscle blood flow (Tappy et al., 1994). Numerous conditions of insulin resistance have been proposed.

Insulin resistance is characteristically associated with truncal obesity, glucose intolerance, hypertension, dyslipidaemia, disorders of blood coagulation and accelerated atherosclerosis (metabolic syndrome or Syndrome X) (Goke, 1998). Substantial evidence suggests that diets high in fat lead to major impairments in insulin action (Storlien et al., 1996). Skeletal muscle insulin action deteriorates under conditions when triglyceride supply is high. In conditions like obesity and increased dietary fat consumption, there are elevations in free fatty acids (FFA). An increased supply of FFA to the liver prevents the

binding of insulin to its receptor and can be considered as one of the factors leading to insulin resistance (Brindley, 1992). This increased supply often exceeds the rate of beta-oxidation and the excess fatty acids are often esterified to triacylglycerol and ultimately secreted from the liver as very low density lipoproteins (vLDL)(Brindley, 1995). Kolterman et al. (1980) reported a predominant insulin postreceptor defect in both liver and peripheral tissues in obese states. In obese subjects, increased upper body fat has been shown to be associated with reduced glucose tolerance, hyperinsulinemia and hypertriglyceridemia (Bjorntorp, 1996). Conversely, significant weight reduction is associated with major improvements in insulin sensitivity.

Not surprisingly, a number of endocrine conditions are associated with insulin resistance. Glucagon, as a counter-regulatory hormone protecting against hypoglycemia, opposed insulin action on hepatic glucose metabolism (Ferrannini et al., 1982). Noradrenaline impedes both insulin-mediated glucose uptake and suppression of lypolisis (breakdown of triglycerides) (Lembo et al., 1994). Physiological concentrations of growth hormone (GH) have been shown to enhance lipolysis and ketogenesis, thus antagonizing the effects of insulin (Gerich et al., 1976). Similar findings have been reported in studies on the effects of the adrenal GCs.

GCs exert profound effects on glucose homeostasis under both normal and pathological conditions. During periods of fasting, cortisol, in concert with other counterregulatory hormones, participates in the maintenance of blood glucose levels by decreasing utilization and increasing production of glucose (Boyle et al., 1991). Stress hormones generally antagonize the actions of insulin (Brindley and Rolland, 1989). Insulin promotes the uptake of glucose by muscle and adipose tissue and stimulates subsequent glycolysis (the breakdown of glucose into pyruvate) (Brindley, 1995). Cortisol produces an insulin resistance in tissues within a few hours and it inhibits insulin-mediated glucose uptake. Cortisol also stimulates hepatic gluconeogenesis, thus increasing glucose

concentrations and opposing the actions of insulin (Brindley, 1995). Part of the substrate provision for gluconeogenesis is supplied by the transport of amino acids from muscles. Cortisol increases the breakdown of protein, glycogen and triacylglycerol in muscle while insulin has the opposite effect. Similarly, insulin is able to decrease lipolysis in adipose tissue, thus decreasing the supply of non-esterified fatty acids (NEFA) and glycerol to the liver. Cortisol increases glycerol release from adipose tissue by producing insulin resistance.

Cortisol is also an important component in increasing very low-density lipoprotein (vLDL) secretion (Brindley, 1995). This has atherogenic implications, since vLDL is metabolized into intermediate and low-density lipoproteins (IDL and LDL). High concentrations of insulin can normally antagonize the stimulatory effects of cortisol on vLDL secretion (Brindley and Salter, 1991); in insulin resistant states, however, the lack of suppression by insulin coupled with increased fatty acid supply is likely to contribute to hypertriglyceridemia. The clearance of IDL and vLDL rely primarily on hepatic LDL receptors. Expression of this receptor and the degradation of LDL is increased by insulin and decreased by cortisol (Brindley and Salter, 1991). Consequently, the increased action of cortisol relative to insulin can potentially produce a hypercholesterolemia.

Another source may be an increased fatty acid synthesis in the liver through the combined effects of insulin and cortisol (i.e. synergism). Cortisol facilitates the actions of insulin in stimulating the synthesis of fatty acids (Al-Sieni et al., 1989) and glycogen and the activity of lipoprotein lipase in adipose tissue (Speake et al., 1986). Taken together, these reactions are involved in energy deposition in the body (the simultaneous release of cortisol and insulin after a meal in humans is probably a signal for energy deposition (Brindley and Rolland, 1989). Thus, there appears to be two general types of interaction between insulin and the GCs. When insulin availability is low, GCs have a catabolic effect in the body and antagonize insulin's actions. When insulin actions are high, GCs have a

general anabolic effect leading to the deposition of energy, increased weight and possibly obesity. In terms of their roles in food intake, Strack et al (1995) showed that GCs and insulin are reciprocal signals for energy balance. The hormones serve opposite roles in the central nervous system, where GCs stimulate and insulin inhibits food intake, and in the periphery, where GCs inhibit and insulin stimulates overall energy storage.

Glucocorticoids and Feeding

Normal Feeding Cycles

In the normal weight rat, total daily caloric intake is tightly regulated and maintained within a narrow range (Anderson, 1988; Le Magnen, 1992). This is evidenced behaviorally by the ability of animals to adjust the amount of calories consumed after dilution or concentration of the diet, so that a constant daily caloric intake is maintained (Le Magnen, 1992). Depending on the requirements, consumption leads to oxidative metabolism for immediate use and/or storage in the body for later utilization. The daily rhythm in energy balance in the rat is generally characterized by a positive energy state during the active period (dark cycle) when there is increased intake and storage of nutrients (Armstrong, 1980; Le Magnen, 1981) and energy storage is accomplished by increased synthesis of protein, glycogen and fat under the control of insulin (Dallman et al. 1993). The negative energy state develops over the course of the inactive period (light cycle) and involves little eating and continued breakdown and utilization of nutrient stores. However, at any time during the 24-hour day, restriction of access to an external energy source, stress or other manipulations can override the circadian rhythms in endocrine activity (Dallman et al., 1993), generated by the suprachiasmatic nucleus (SCN). Conversely, the daily rhythm in feeding, can be reversed by infusions of insulin during the day and infusions of epinephrine during the night, when there is ad-lib access to food (Bray et al., 1989; Bray et al., 1990; Le Magnen, 1992).

Nutrient ingestion and metabolism are not uniformly distributed across the 12-hour feeding period in the rat. Feeding is characterized by a bimodal distribution, with peaks during the initial and final 3-4 hours of the dark period (Armstrong, 1980; Le Magnen, 1981; Johnson et al., 1986). These bouts of feeding have different characteristics and are associated with different metabolic processes (Tempel and Leibowitz, 1994).

a) Intake and metabolism of protein:

In rats and humans, protein intake is maintained at a constant 10-20% of total caloric intake regardless of the protein concentration of the diet (Johnson et al., 1986; De Castro, 1987). In animals on pure macronutrient diets, the ingestion of protein remains fairly constant across the active feeding period, exhibiting a small increase towards the latter portion of the night. (Tempel and Leibowitz., 1989; Shor-Posner et al., 1991; Shor-Posner et al., 1994). Protein consumed in the diet is enzymatically hydrolyzed in the alimentary tract and passes into the blood as free amino acids that mingle with amino acids coming from the tissues (Felig et al., 1995). Amino acids are absorbed and incorporated into muscle tissue. While some body protein breakdown and replenishment may occur throughout the day, absolute protein stores remain relatively stable, with only small amounts of ingested protein geared towards storage and any excess eliminated as urinary nitrogen (Felig et al., 1995).

b) Intake and metabolism of carbohydrate

The maintenance of carbohydrate stores is critical towards the end of the light/dark cycle (the end of the inactive period), when nutrient reserves are at their nadir, and energy demands are rapidly increasing (Armstrong, 1980; Le Magnen, 1981). Storage and ingestion of carbohydrates exhibit dramatic fluctuations across the day. Glucose is stored as glycogen, primarily in the liver, muscle and brain, and these glycogen stores are mostly

small, short-term stores used primarily to maintain blood glucose levels (Le Magnen, 1992). Thus they fluctuate widely across the day, from a state of near depletion prior to the initiation of the feeding cycle, to full restoration in the second half of this cycle when glucose levels tend to rise. When given a choice of foods, normal weight animals as well as humans, select a diet rich in carbohydrates, at the start of the feeding cycle (Shor-Posner et al., 1991, 1994). In rats the first meal of this cycle may contain between 40-50% (and as much as 100%) carbohydrate, and the carbohydrate consumed during the first few hours of the nocturnal period may account for up to 50% of the animals total daily intake (Tempel and Leibowitz, 1994).

At this point in the cycle, the organism is prepared to absorb, metabolize and store carbohydrates, at the same time as the peak in pancreatic insulin release, glucose absorption and tolerance and glycogenic enzyme activity in the liver (Anderson, 1980; Van Cauter et al., 1989). Glycogenolysis and gluconeogenesis in the liver raises blood glucose levels for immediate oxidation during this first period of increased activity. Then with a further rise in glucose availability, it allows liver glycogenesis to replenish carbohydrate stores geared for future use (Abbot et al., 1988; Van Cauter et al., 1989). Carbohydrate consumption then drops after the first few hours and the final meals of the cycle are primarily fat and protein. Moreover, since liver glycogen and adipose tissue stores are replenished, the bout of late feeding is geared towards filling the stomach where it can be stored for several hours before being used (Armstrong et al., 1978; Strubbe et al., 1986).

c) Intake and metabolism of fat

Dietary fats are slowly absorbed from the gut. Rather than being immediately oxidized, they are taken up and stored in adipose tissue, the largest fuel store of the body. Fat is most strongly preferred, in both animals and humans, during the middle to late hours (De Castro, 1987; Shor-Posner et al., 1991), and a sharp rise in fat ingestion can be seen between the third and sixth hour of the feeding cycle. The anticipatory nature of the feeding

during these later hours, when the body's nutrient stores are almost replenished, provides nutrients for storage in the gastrointestinal tract, which retains the food for several hours and supplies the nutrients to be utilized during the early hours of the inactive period.

When stomach stores are exhausted, the body's own reserves of fat, carbohydrate and to a lesser extent, protein, are called upon, broken down and utilized to meet energy requirements. During the middle and later periods of the light cycle, essential nutrients are obtained primarily from lipolysis in adipose tissue. The breakdown of fat stores releases free fatty acids (FFA) into the blood, which can be used as an energy substrate by most tissues of the body except the brain (Felig, 1995). Lipolysis also releases glycerol, which can be used by the liver for gluconeogenesis.

Effects of Glucocorticoids on Metabolism

Glucocorticoids were named for their glucose-regulating properties and have extensive influences on carbohydrate, lipid, protein and nucleic acid metabolism. Although these steroids are secondary to insulin in regulating glucose metabolism in humans, they influence blood sugar levels and play a protective role against glucose deprivation (Felig et al., 1995). The latter role provides an excellent conceptual framework for considering many of the coordinated actions of the GCs on carbohydrate, lipid, protein and nucleic acid metabolism.

Carbohydrate Metabolism

GCS increase glucose production by enhancing hepatic gluconeogenesis, by releasing gluconeogenic substrate from peripheral tissues, by enhancing the ability of other hormones to stimulate gluconeogenesis (Steele, 1975; Exton, 1979; Margolis and Curnow, 1983) and decrease glucose transport (Horner et al., 1990; Leighton et al., 1991; Virgin et al., 1991). In fed, ADX-ed animals, basal gluconeogenesis is not impaired but there is an impaired response to glycagon or catecholamines. In fasted or diabetic animals, ADX

results in a net reduction in hepatic gluconeogenesis, which is reversible with corticosterone administration (Dallman et al., 1994). Thus GCs are required to maintain gluconeogenesis in fasting and insulin deficiency.

Essentially every step in the gluconeogenic pathway is influenced by GCs. They increase total hepatic protein synthesis and increase several of the transaminases, especially alanine aminotransferase (Felig et al., 1995). These steroids also increase the activity of phosphoenolpyruvate carboxykinase (PEPCK) and possibly glucose-6-phosphatase (Coufalik and Monder, 1981). GCs also play a permissive role by increasing the sensitivity of the liver to the gluconeogenic actions of glucagon and the catecholamines. Bilateral adrenalectomy has long been known to impair an animal's ability to maintain normal glucose homeostasis under prolonged starvation (Hers, 1985). Perfused muscle from adx rats shows a net decrease in the release of amino acids, which is reversed by GCs. The steroids decrease protein synthesis and can increase protein breakdown in several tissues such as muscle, adipose and lymphoid, resulting in an increased release of amino acids (Felig et al., 1995).

GCs also increase gluconeogenic substrates by increasing glycerol release from fat cells (by stimulating lipolysis) and lactate release from muscle. Lipolysis also provides free fatty acids, which cannot themselves contribute to a net increase in gluconeogenesis but can provide energy for gluconeogenesis and spare other substrate that can be converted into glucose.

GCs also inhibit glucose uptake and metabolism in peripheral tissues. There is a direct inhibition of glucose uptake in adipose tissue (Fain, 1979), fibroblasts (Carter-Su and Okamado, 1985), certain lymphoid cells and fat cells (Munck, 1979; Fain, 1979). Two recent reports suggest that GCs may do the same in the brain. In numerous brain regions, adx increased and GCs decreased local cerebral glucose utilization (Kadekaro et al., 1988).

Horner et al. (1990) have shown that GCs inhibited 25% of glucose transport in hippocampal neurons and glia.

Lipid metabolism

GCs increase lypolisis and plasma free fatty acid levels (Tomas et al., 1979; Fain, 1979; Gaca and Bernend, 1974). Lipolysis and FFA release are increased by the GC-induced decrease in glucose uptake and metabolism that reduces the glycerol production necessary for reesterification of fatty acids. The steroids also stimulate lipolysis by increasing the efficiency of other lipolytic factors, such as catecholamines. The increase in free fatty acid release and the possible augmentation of hepatic conversion of FFAs to ketones by GCs cause a tendency to ketosis. GCs can also cause increases in very low-density lipoproteins (vLDL), LDL and HDL, with consequent elevations of total triglyceride and cholesterol levels (Felig et al., 1995).

Protein and nucleic acid metabolism

GCs affect protein synthesis and breakdown (Loeb, 1976; Baxter, 1978). The steroid stimulates protein synthesis in the liver, inhibits synthesis and stimulates breakdown in many peripheral tissues such as muscle, skin, adipose, lymph node and fibroblast. This pattern may reflect the body's need to provide substrate for hepatic gluconeogenesis from "less essential" tissues, such as muscle, and to decrease substrate utilization while sparing certain other tissues (like the brain and heart).

Effects of Glucocorticoids on Food Intake and Body Weight

The HPA axis exhibits a strong diurnal rhythm, with peak activity occurring at the start of the active period, the dark phase for rats and light phase for humans (Krieger, 1979). This peak in circulating B levels at the onset of activity is preceded by a 3-4 hour period involving increased CRF gene expression and peptide levels in the paraventricular nucleus (PVN) of the hypothalamus, followed by a rise in pituitary ACTH release (Krieger, 1979;

Dallman, 1984; Dallman et al., 1987; Kwak et al., 1992). This rhythm is controlled, in part, by the feedback actions of B, on both type 1 and 2 receptors (Keller-Wood and Dallman, 1984; Gustafson et al., 1987; Sawchenko, 1987; Spencer et al., 1990; De Kloet, 1991), and it involves additional brain areas, including the SCN and hippocampus (Makara et al., 1981; 1986; Sapolsky et al., 1990; Jacobson and Sapolsky, 1991; Herman et al., 1993; Spencer et al., 1993). The feeding process itself has a major role in determining the endogenous rhythms of HPA activity (Krieger, 1979; Honma et al., 1983; Honma et al., 1984; Dallman et al., 1987). This peak in B serves an activational purpose: at a time of low energy stores and increased risk, it prepares the organism for physical activity and enhanced vigilance during the awake cycle (Miller et al., 1990).

In both humans and rats, there is an anticipatory increase in ACTH and corticosteroid secretion that precedes the onset of the daily feeding cycle (Krieger et al., 1971; Quigley and Yen, 1979; Brandenberger et al., 1982; Follenius et al., 1992; Honma et al., 1992; Bligh et al., 1993). When light cycles are shifted with feeding permitted ad lib (light: dark period reversal), the feeding, adrenocortical and other endogenous rhythms reverse (Dallman et al., 1994). Exposure of rats to constant dark results in free-running rhythms in feeding and plasma B, whereas exposure to constant light causes both feeding and B to become arrhythmic (Morimoto et al., 1977). Rats also secrete B after beginning to eat under ad lib feeding conditions (Dallman et al., 1994). In cannulated rats which were sampled for B at regular intervals, particularly in the hours just before and after lights out, secretory episodes of B were best correlated with eating associated activity (Shiraishe, 1984; Hiroshige et al., 1986). In those studies a sharp peak in B followed the onset of a meal by 15 minutes, similar to the timing of the meal-associated cortisol response observed in humans. In rats fasted for 24 hours, Dallman's group confirmed that provision of food elicited rapid increases in plasma B as well as glucose and insulin (Dallman et al., 1989). In rats fasted for 1.5 hours in the AM and then trained to eat a 4 gram test meal within 3.5

minutes, Steffens et al. (1986) showed that glucose, insulin, epinephrine and FFA concentrations all rose at the onset of a meal.

Adrenalectomized rats eat with a normal or exaggerated nocturnal rhythm (Bellinger, 1979); however, when fed standard lab chow, their total daily food intake is reduced (by approximately 20-30% in calories) and they gain weight more slowly than intact rats with adrenals (Cohn et al., 1955; Bray et al., 1990). Provision of B in very low doses (steady-state B up to approximately 3 μg/dl) to adx rats restores feeding to normal (Dallman et al., 1989). Because the circulating levels of B required to restore feeding to normal are so low, one can assume that the effect is mediated by association of the steroid with the high affinity type 1 corticosteroid receptor (King, 1988). In rats fed ad libitum, there is some debate over the effects of ADX on macronutrient preference and calorie intake. Leibowitz's group has shown that in ADX rats fed pure macronutrient diets, the consumption of carbohydrate and fat is most dramatically and consistently reduced, although a predominant effect on fat intake may occur in obese rats with a strong preference for this nutrient (Castonguay et al., 1984) and variable changes in protein intake can be seen (Bligh et al., 1993). Adrenalectomy disrupts carbohydrate as well as fat and protein metabolism. It produces a severe depletion of glycogen stores and decreased fat deposition, and under conditions of food restriction and stress, it leads to an inability to mobilize fat and protein reserves for maintaining essential carbohydrate nutrients (Seyle, 1936; Margolis and Curnow, 1983; Foss et al., 1987; Bligh et al., 1993, Bray et al., 1992).

Leibowitz and colleagues have tested the effects of systemic injections or implants of crystalline steroids over the PVN at the time of lights out on calorie intake and macronutrient preference during the succeeding 60 minutes in adx and intact rats (Tempel and Leibowitz, 1989). In intact rats, aldosterone (a type 1 corticosteroid receptor agonist) caused increased calorie and fat consumption, whereas B and the type 2 receptor agonist, RU28362 and dexamethasone had no effects when administered either systemically or

directly over the PVN. In adx rats, B, aldosterone and RU28362 administered by both routes, significantly increased carbohydrate consumption, whereas only aldosterone increased consumption of fat (Tempel et al., 1992). These results suggest, again, that the first meal intact rats take in at night (at the time of peak basal B levels) is predominantly composed of carbohydrate.

The question of the long-term effects of ADX remains. The major effect across days and weeks of both B and aldosterone administration is to normalize food intake and to restore fat intake and it's deposition (Kumar et al., 1988; Castonguay, 1991; Devenport et al., 1991). Treatment with dexamethasone or RU2862 did not restore food intake or body weight of ADX-ed rats to normal (Devenport and Stith, 1992). The discrepancy between single, acute injections of type 2 agonists and chronic administration of these on feeding may reside in the apparent inhibition of eating by insulin. A single injection of a type 2 agonist could act on feeding before the time it takes to induce elevation in insulin levels. Devenport and Thomas (1990) tested daily macronutrient preferences in sham adrenalectomized and adx rats with or without infusion of aldosterone (25 or 125 μg/kg/day). The low dose of ALDO restored intake, body weight gain, and food efficiency to normal in adx rats. Adrenalectomized rats decreased fat intake by 60% and carbohydrate intake by 25% compared with intact rats and ALDO restored these to normal.

Replacement studies in adx animals indicate that the impact of B administration is greatly determined by dose. Twenty-four hour caloric intake and body weight gain can be restored by low, chronic doses of B which raise blood levels to 1-2μg% (Kumar et al., 1988; Devenport et al., 1991). These low B levels also promote the deposition of fat (Steele, 1975; Fain and Czhech, 1975; Bray et al., 1990; Felig et al., 1995). However, they are insufficient in restoring the initial carbohydrate meals of the feeding cycle (Kumar et al., 1988; Tempel et al., 1991; Tempel et al., 1992) as well as promoting fat or protein catabolism (Kumar and Leibowitz, 1988; Devenport et al., 1989). These latter effects

require moderate B levels, in the range of 5-10μg% (Flatt, 1978; Devenport et al., 1989). At this concentration, the adrenal steroid replenishes glycogen stores in the liver and muscle, through the stimulation of gluconeogenic enzyme activity and glycogen synthase activity (Felig et al., 1995) and endogenous B in adrenal-intact rats is positively correlated with carbohydrate intake in the early dark hours, at least in the short term (Tempel and Leibowitz, 1994). Higher levels of circulating B (>10μg%) maintained across the light: dark cycle have additional actions that are catabolic in nature. That is, under conditions such as chronic stress or food restriction, this steroid, in both ADX and adrenal-intact animals, has potent effects designed to make glucose available (Munck et al., 1984; Devenport et al., 1989; Felig et al., 1995).

Glucocorticoids, restricted feeding and fasting

With the exception of very young, rapidly growing rats, there does not seem to be an effect of an acute 24 hour fast on basal B (Shiraishi et al., 1984; Dallman et al., 1989). Over a similar time course, presentation of water or food for 30 minutes to 4 hours per day alters the basal rhythm in HPA activity such that peak activity occurs for a variable time, just before provision of food or water (Dallman, 1984). Honma et al. (1983; 1984) have correlated the duration of the feeding period and the amount of food ingested, to the relation of the rhythm in B to the times of feeding and the light cycles. Rats fed for 0.5 or 2 hours/day beginning at mid-light cycle, after 14 days exhibited peak B levels in the sample before feeding, although a second rise at lights out was usually observed. By contrast, rats fed for 6 hours per day did not exhibit a prefeeding rise in B and showed only the normal rise in B before dark onset. Only the rats fed for 6 hours ate as many calories as ad lib-fed rats; the other restricted groups ate, and weighed, significantly less. The conclusions drawn were: both periodic feeding and a distinct state of fasting are required to demonstrate the prefeeding peak in B and that after prolonged fasting, the mean daily B levels in rats allowed to eat were determined by the amount of food ingested during the permitted feeding period.

Adrenalectomized rats exposed to restricted feeding do not increase their food intake during the period of feeding as do intact animals; rather, even with low-dose corticosteroid treatment they decrease the amount of food ingested over subsequent days and die, unless they are fed a calorically-dense, high-fat diet (Kaul and Berdanier, 1975).

With increasing duration, fasting elevates plasma hormones of the HPA axis in many species. Akana et al. (1994) demonstrated that when rats are not allowed to eat during the night, they exhibit a pattern of increased ACTH and B secretion during this time, which mirrors the normal pattern of feeding in rats fed ad lib, suggesting that secretion of hormones in the HPA axis serves as a default pathway to feeding. The effects of starvation on HPA axis function is observed primarily during the trough of the rhythm in both humans and rats (Morimoto et al., 1977; Kato et al., 1980; Honma et al., 1983; Fichter et al., 1986; Vance and Thorner, 1989).

High fat diets and GCs

High-fat diets contribute to insulin resistance (Reaven et al., 1967; Storlien et al., 1986), impaired glucose metabolism (Glueck et al., 1969), type II or non-insulin dependent diabetes mellitus (NIDDM) (Himsworth, 1935; Kolterman et al., 1979; American Diabetes Association, 1987), stroke and coronary artery disease (Lipid Research Clinics Program, 1984). Dietary fat is associated with increased low-density-lipoprotein (LDL) concentrations and the elevated triglyceride levels in very low-density lipoproteins (vLDL). Fat from the diet not only lowers glucose uptake but also stimulates inappropriate glucose production (Anderson and Seiling, 1985), resulting in elevations in both insulin and glucose (Reaven et al., 1967). High fat diets decrease the number of insulin receptors in liver, skeletal muscle and adipose tissue, decrease glucose uptake into skeletal muscle and adipose tissue and decrease hepatic glycolysis and glycogen synthesis. Glycogen accumulation and glucose oxidation are lower with high-fat diets and the rate of gluconeogenesis is increased in the liver (Anderson, 1982), a common problem for many diabetics. In sum, high-fat diets

are associated with a Syndrome X-like state that includes hypertriglyceridemia, decreased high-density lipoproteins (HDL, or "good cholesterol"), high LDL and vLDL, abnormal glucose production, hyperinsulinemia and insulin resistance (Reaven, 1988).

High fat diets also have effects on HPA activity, elevating adrenal GC production (Brindley et al., 1981; Hulsmann, 1978; Pascoe et al., 1991; Trottier et al., 1998; Tannenbaum et al., 1997; Kamara et al., 1998; see also chapters 2-5). Increased GCs also stimulate secretion of triglycerides from the liver in vLDL (Bartlett and Gibbons, 1988; Mangiapane and Brindley, 1985). GCs also decrease levels of lipoprotein lipase, which can exaggerate hypertriglyceridemia (Taylor and Agius, 1988). Normally, most LDL formed after the degradation of vLDL is removed from circulation by LDL receptors; the binding and degradation of LDL by rat hepatocytes are decreased by DEX, which could result in an elevation in LDL levels (Goldstein and Brown, 1977).

A prolonged excess in GC levels leads to an alteration in the balance between GCs and insulin. Elevated GCs antagonize most of insulin's actions and result in increased basal and glucose-stimulated insulin levels and pancreatic beta cell hyperplasia (Lenzen and Bailey, 1984; Martin-Sanz et al., 1991). Insulin inhibits the secretion of triacylglycerol, phospholipids, cholesterol ester, and apolipoproteins A and B associated with vLDL (Brindley et al., 1988).

Stress and General Feeding

Feeding in response to acute stress

While both pharmacological and dietary manipulations can affect HPA axis function, the reciprocal relationship also exists. Numerous groups have examined the effects of acute stressors on food consumption and body weight. Some of the stressors that have been used include tail pinch, restraint, immobilization, and acute treatment with CRF. Krahn et al. (1986) examined the effect of one hour of restraint on feeding behavior in food

deprived rats. Restraint significantly reduced food intake relative to unrestrained controls. This effect of restraint stress on feeding has been replicated by a number of groups (Kennet et al., 1985a;b; Donohoe et al., 1987); in fact, Shibasaki et al. (1988) found that 90 minutes of restraint stress reduced food intake by rats to approximately 60% of control rats levels. In addition, restraint stress-induced reductions in feeding were partially dependent on the duration of restraint: 30 minutes of restraint was sufficient to reduce food intake and 60 and 90 minutes of restraint caused an even greater reduction in food intake relative to rats restrained for 30 minutes. Heinrichs and Koob (1992) found that restraint stress significantly reduced the intake of unfamiliar food in animals that had been protein deficient. Zylan and Brown (1996) found that 20 minute immobilization stress significantly reduced food intake in both males and females, although females did not consume more food with repeated stressor exposure (chronic and repeated stress effects on food intake will be discussed below). Ely et al. (1997) found that acute restraint did not produce any alterations in consumption of a sweet snack. Conversely, tail-pinch stress has been shown to cause hyperphagia (Robbins et al. 1977; Wallach et al., 1977; Morley et al., 1983; Kalra and Kalra, 1990; Heinrichs et al., 1992). Therefore, these results suggest that both the type of stressor and type of food presented may have an impact on the feeding response to acute stress.

Feeding in response to chronic/repeated stress

Chronic exposure to a variety of stressors of a certain severity has been shown to decrease food intake and body weight gain in the rat. Marti et al. (1994) subjected adult male rats to various chronic stressors of differing intensities and for different periods of daily exposure. While a mild stimulus, such as one minute of daily handling for 27 days, had no effect on food intake and body weight, chronic restraint slightly reduced food intake and body weight gain and chronic, daily immobilization produced the greatest decrease in food intake and body weight gain (Marti et al., 1994). The responses were similar during

the entire exposure period because the changes in body weight gain and food intake were of the same extent 1 or 4 weeks after the beginning of treatment. Thus habituation to these chronic stressors was not apparent in this study. In a second experiment, rats were exposed to 2-hour immobilization, every day for 14 days (Marti et al., 1994). Food intake was most dramatically decreased following a single 2-hour session (acute effects), but remained decreased on subsequent days of exposure to isolation stress. No major changes in the circadian rhythm of food intake was found in response to chronic immobilization, but the percent of food eaten by stressed rats between 0400-0800h was always lower than that of control rats. The authors also assessed the influence of the daily duration of exposure to the same stressor: while adrenal weights were greater in rats subjected to 240 minutes of immobilization stress when compared to rats exposed to 15 and 60 minutes of immobilization, all rats consumed similar amounts of food and showed similar reductions in body weight (Marti et al., 1994). In the study by Ely et al. (1997) animals that were restrained for one hour per day for 5 days per week for 50 days showed an increased ingestion of sweet food when compared to unstressed controls. Heinrichs et al. (1992) found that repeated tail pinch (once per day for 4 days) resulted in increased food consumption with each successive day and a decreased latency to eat between the first testing day and the fourth. Repeated cold stress resulted in increased food intake with decreased body weight gain (Kawanishi et al., 1997). In addition, the diurnal variation in body weight decreased. Kant and Bauman (1993) found that animals exposed to chronic, intermittent footshock decreased their food intake of both low and high sucrose pellets, although there was a greater decrease in pressing for the low sucrose pellet than the high sucrose pellet. Zylan and Brown (1996) found that with repeated immobilization stress, both males and females continually increased their food intake with each successive session and females, in particular, showed an increase in food intake from the third to the fourth sessions, while males stabilized their intake between the third and last session.

Stress and macronutrient selection

While few studies have examined the relationship between stress and macronutrient selection in the rat, most have tested varying levels of corticosterone (both in basal and stress level ranges) on the preference for fat, protein and carbohydrate. Leibowitz's group has examined the relationship between macronutrient selection and various hormones and neurotransmitters in rats. In fact, rats exhibit profound individual differences in their natural patterns of nutrient intake. Shor-Posner et al. (1991) found that self-selecting albino rats could be subdivided into three distinct subpopulations according to their preference for carbohydrate, fat or protein. They also found that the high-fat preferring rats have a higher body weight, while those preferring the high carbohydrate have the lowest body weight (Shor-Posner et al., 1991). Finally animals show a distinct macronutrient preference at the onset of the active (nocturnal) cycle but are generally similar when during the middle dark phase when diet preferences are minimal and also during the late dark phase, when most rats consume a diet rich in protein and fat relative to carbohydrate (Shor-Posner et al., 1991).

Very different patterns of intake and metabolism exist for the three macronutrients as well. Ingestion of carbohydrate, fat and protein partially depends on the level of circulating corticosterone. Numerous studies giving GR and MR agonists and antagonists peripherally have revealed that in general, the selective type 1 receptor agonist, ALDO, is effective in restoring daily caloric intake and body fat deposition and its predominant action is on the ingestion and metabolism of fat (Devenport and Thomas., 1990; Tempel and Leibowitz, 1994), although it can affect all macronutrients. This preferential role in fat balance is most clearly seen during the later hours of the feeding cycle (Tempel and Leibowitz, 1989; Tempel et al., 1991). Further support for the specificity of ALDO in fat ingestion and metabolism, is its inability to produce normal patterns of carbohydrate ingestion (Tempel and Leibowitz, 1989) and the inability of the type II agonist, RU48362, to cause changes in fat consumption. Administration of RU48362, as well as B, at dark onset,

reveal a strong stimulatory effect on carbohydrate intake in rats (Tempel et al., 1992). It is also ineffective in potentiating carbohydrate intake during the light period and, like B, has little effect towards the end of the nocturnal feeding cycle (Tempel et al., 1993), when endogenous B levels, type II receptor occupation and carbohydrate feeding are normally low.

Stimulation of central adrenal steroid receptors have similar affects on macronutrient consumption. After ventricular administration (i.c.v), the stimulatory effect of B on daily caloric intake in ADX rats occurs at considerably lower doses than are required when peripherally administered (Green et al., 1992) and an enhancement of daily food intake can also be seen with ventricular injections of ALDO (White et al., 1991). With steroid implants directly into the PVN, there is evidence to suggest the involvement of PVN type II receptors at mediating the natural surge in carbohydrate ingestion at the onset of the feeding cycle (Shor-Posner et al., 1991). The type I agonist, ALDO has no effect on carbohydrate intake but does have a tonic stimulatory action on fat intake (Tempel and Leibowitz, 1989). The reduced fat ingestion detected in ADX rats is reversed by PVN implants of ALDO and ALDO potentiates fat ingestion equipotently at all times of the feeding cycle (Tempel and Leibowitz, 1989).

There is currently no literature describing the relationship between acute and/or chronic stress and macronutrient selection rats. However, if one was to extrapolate from the peripheral and PVN implant data described above, it would seem reasonable to assume that stress levels of B would potentiate the ingestion of carbohydrate and this potentiation would be seen at both the onset and conclusion of the feeding cycle. With chronic stress, however, the situation is less obvious. Chronic elevations in B across the light-dark cycle can cause both weight gain (and ensuing obesity) and weight loss. In chronically stressed animals, the main effect of high B is to make glucose available. These catabolic actions involve an increased breakdown of the body's fat and protein stores, resulting in a decline in

body weight (Devenport et al., 1989). On the other hand, all types of obesity syndromes are attenuated, abolished and/or prevented by ADX surgery and are restored by steroid replacement (Bray et al., 1990). Moreover, the fat deposition in obese animals is positively correlated with circulating B levels (Tempel and Leibowitz, 1994). In addition, obese animals exhibit a disturbed daily rhythm of feeding and circulating B levels which are elevated during the inactive period (King, 1988). This same elevation in AM B is seen in chronically stressed rats (Scribner et al., 1991). Thus, in chronic stress states, there is an initial catabolic role for B; here high B levels defend carbohydrate stores by breaking down fats and protein. This may cause a decline in body weight. With continued exposure to B, a compensatory increase in insulin may occur thereby antagonizing the catabolic actions of B (Brindley and Rolland, 1989). In addition to stress, there are numerous other HPA-related factors that may have an impact on the development of eating patterns and the metabolic systems governing body weight.

Early Environmental Influences on Eating and Body Weight

The early neonatal environment has long-term influences on the development of the HPA response system. Animals separated from their mother during the neonatal period may develop permanent alterations in behavioral and endocrine responses to stressors (Francis et al., 1996; Levine, 1994; Meaney et al., 1994). The length of separation is critical in determining the development of later HPA responses to stressors: pups exposed to daily, brief (15 minutes per day) periods of separation (i.e. neonatal handling or H) for the first 2-3 weeks of life show a reduced ACTH and B response to stress when tested as adults. Handled animals show a smaller stress-induced rise in ACTH and B and a faster return to baseline levels following the termination of the stressor (Meaney et al., 1988) when compared to their non-handled (NH) counterparts, despite a lack of a difference in basal ACTH and B. H rats also show an increased expression of GR in the hippocampus and

frontal cortex, which may account for their enhanced negative feedback efficacy (Meaney et al., 1996).

Early neonatal manipulations can cause alterations in anxiety and cognitive behaviors. H animals exhibit less anxiety/fear on the elevated plus maze and hyponeophagia paradigm (Bodnoff et al., 1987; Ferre et al., 1995), Posolt Swim test (Hilakivi-Clarke et al., 1991), increased exploration in the open field (Levine et al., 1967) and are spared the age-associated decline in spatial learning (Meaney et al., 1988). Longer periods of separation, however, have very different effects on these parameters. When rats are separated from their mothers for 3 hours per day for the first 2-3 weeks of life (maternal separation or MS), they show increase HPA responsivity to stressors when tested as adults (Ladd et al., 1996; Levine et al., 1992; Meaney et al., 1996). MS rats display greater acute stress-induced rises in ACTH and B and decreased hippocampal and cortical GR binding (Vasquez et al., 1996). Maternal separation is associated with increased CRH receptor binding and increased hypothalamic CRH mRNA expression (Plotsky and Meaney, 1993).

There is some current clinical evidence suggesting that early life experiences may also influence eating behavior and may be an important factor contributing to the later development of eating disorders, such as anorexia nervosa (Kinzl et al., 1994; McElroy and Keck, 1995). Numerous studies link anorexia nervosa or bulimia to parental dysempathy, neglect or hostility (Wonderlich and Mitchell, 1992) and to alarming rates of sexual abuse in childhood (Everhill and Waller, 1995). Kent et al. (1999) recently found that emotional abuse suffered in childhood was the strongest predictor of unhealthy eating attitudes in adult women. Wonderlich and Mitchell (1997) also found that childhood sexual abuse was a strong risk factor for bulimia nervosa; however they also note that psychological factors such as depression, body mass index and weight dissatisfaction are also influential in the relationship between childhood sexual abuse and eating disorders.

The evidence from animal studies suggesting a link between the early environment and eating behaviors later in life has been much less extensive. Vallee et al. (1996) examined the long-term effects of both prenatal stress and neonatal handling on metabolic parameters in adult rats. They reasoned that since both prenatal stress and neonatal handling cause alterations in the development of basal and stress-induced HPA axis in adult rats, and since feeding behavior, body weight and glucose dynamics are tightly regulated by HPA activity, then it would follow that those manipulations causing alterations in HPA axis activity could also impact on metabolic functioning. Valle's group (1996) found that the effects of prenatal stress and perinatal handling had strikingly opposite effects, namely that the handled rats show a reduced B response following the termination of stress while prenatally stressed rats show an overall increased B response to restraint. Handled animals had higher body weights and lower basal blood glucose level when compared to both prenatally stressed and control rats while prenatally stressed rats showed lower body weights when assessed at 5 months of age. Prenatally stressed rats had significantly higher blood glucose levels when compared to control and handled rats; handled and control rats had similar basal glucose profiles. Finally, while water intake was similar across all groups, food intake was strikingly different in handled, control and prenatally stressed rats. Prenatally stressed rats consumed significantly less food compared to the other rats while handled rats ate the most. McIntosh et al. (1999) examined the impact of early life experiences on appetitive responses. In addition to examining handled animals they also looked at maternally separated (MS) and non-handled (NH; similar to the control animals in the Valle et al. study described above). At weaning, the MS animals weighed significantly less than both the H and NH animals; the difference between the H and MS was more robust and persisted throughout the experiment (until day 62). Both male and female handled rats consumed more of a palatable "snack" than their NH counterparts; however, the feeding suppressant response to various satiety peptides (CCK, bombesin and amylin) tested was similar.

In this respect it is interesting to consider the role of the HPA axis in both depression and eating disorders, since these may share a similar etiology. During the underweight phase of anorexia nervosa there are abnormalities in neuroendocrine function, including sustained hypercortisolism, increased central CRH, normal ACTH and altered regulation of plasma and CSF AVP (Licinio et al., 1996). There is also a decrease in GR mononuclear leukocytes (Kontula et al., 1982). The plasma ACTH response to CRH is significantly blunted in underweight anorexics (Gold et al., 1986) and is negatively correlated with basal plasma F levels. This data suggest that the pituitary corticotroph cell responds appropriately to GC negative feedback and that the hypercortisolism reflects a defect at or above the hypothalamus (resulting in increased CRH secretion). None of the anorexics in Gold et al.'s study (1986) had normal suppression of plasma F after DEX administration, suggesting that the adrenal cortex in anorexics has grown hyperresponsive to ACTH during the course of long-standing hypercortisolism. In patients with bulimia nervosa, urinary free F, basal ACTH and CRH-stimulated ACTH and cortisol were normal. Like anorexics patients, depressed patients manifest hypercortisolism and a markedly attenuated ACTH response to exogenous CRH. Patients with depression also have increased levels of CSF CRH (Nemeroff et al., 1984). Hence, the proposed hypersecretion of CRH in anorexia nervosa may be a finding independent of weight loss. In this regard, anorexia nervosa and major depression may have many clinical, pathophysiologic and genetic features in common and some investigators have hypothesized that both illnesses lie on a broad continuum of depressive spectrum disorders (Cantwell et al., 1977).

Therefore GCs have widespread functions, ranging from their role in the stress response to their critical functions in intermediary metabolism. Because of their highly catabolic nature, prolonged exposure to GCs accelerates the progression of disease. A large body of literature has proposed an integral role for the GCs in the acceleration of the aging process. The consequences of cumulative exposures to GCs in accelerating "aging" are an increased risk for a variety of common, age-related pathologies and an increased potential

for damage to central nervous system pathways involved in regulating the containment of circulating GCs. This could result in a synergistic cascade in which the resulting prolonged elevations in GCS further increase risks for many pathologies as well as potentiating damage to HPA negative feedback mechanisms.

Glucocorticoids and the Aging Process

Research on aging has emphasized average age-related losses and neglected the substantial variation that occurs in older individuals (Rowe and Khan, 1987). A major component of many age-associated declines can be explained in terms of the prenatal and early neonatal environment, life style, habits, diet, and an array of psychosocial factors extrinsic to the aging process. For years, for example, it was believed that HPA activity increased with age, and subsequent neuronal degeneration and cognitive impairment was inevitable. Numerous studies, however, have suggested that increased HPA activity is not an inevitable consequence of aging in humans, primates and rats and that environmental manipulations may explain one possible source of variability in aging studies. Nonetheless, it is imperative to understand how elevations in GCs can increase neuronal vulnerability to age-related damage (Porter and Landfield, 1998) and how increased GC levels may contribute to other pathological conditions, such as glucose intolerance, insulin insensitivity and Type II diabetes (Brindley and Rolland, 1989).

HPA Activity in the Aged Rat

While the GCs are an integral and adaptive component of the stress response, prolonged exposure to them can be highly damaging. During stress, GCs serve to increase blood glucose levels, and suppress inflammatory responses (Meaney et al., 1991). Highly catabolic GCs produce lipolysis, increasing the level of free fatty acids, glycogenolysis, increasing blood glucose and protein catabolism, which increases amino acid availability as substrates for gluconeogenesis (Felig et al., 1995). Immune processes are suppressed, which serve to protect the animal against the occurrence of inflammation at a time when the

animal may need to be mobile. In addition, elevated GCs are essential for replacement of plasma volume and protein content after blood loss, particularly in previously fasted animals (Pirkle and Gann, 1976). Thus in response to an acute threat, increased GCs are essential for survival.

Chronic exposure to GCs, however, can be highly damaging to the organism. In addition to a general suppression of anabolic processes, prolonged GC exposure can lead to decreased insulin sensitivity, muscle atrophy, steroid-induced diabetes, hyperlipidemia, hypertension, hypercholesterolemia, arterial disease, impotency, amenorrhea, immunosuppression and impaired tissue growth and repair (Felig et al., 1995). Evidence has been accumulating steadily for 20 years that excessive GCs promote neuron aging and/or loss in rodents (Landfield et al., 1978, 1981; Meaney et al., 1988; Landfield and Elridge, 1994; Sapolsky, 1992; Reagan and McEwen, 1997).

Increased levels of GCs can occur with aging in the rat; increases in plasma ACTH (Tang and Phillips, 1978; Meaney et al., 1992) and corticosterone concentrations (Hess and Riegle, 1970; Sencar-Cupovic and Milkovic, 1976; Landfield et al., 1978; Brett et al., 1983; Sapolsky et al., 1983; DeKosky et al., 1984; Meaney et al., 1992) have been reported. In addition to the age-related changes in basal HPA function, senescent animals also exhibit an exaggerated HPA response to stress. While peak HPA responses remain largely unaffected, corticosterone levels remain elevated for significantly longer periods of time following the termination of the stress when compared with young adult animals (Sapolsky et al., 1986). This decreased GC inhibition of HPA activity is, in turn, associated with a loss of corticosteroid receptors in certain brain regions that mediate negative feedback effects of circulating GCs. Specifically, there is a decrease of both GR and MR in the hippocampus of aged rats (Sapolsky et al., 1983; Ritger et al., 1984; Meaney et al., 1988; 1992; Reul et al., 1987) a critical site for GC-negative feedback (reviewed earlier). Together, these findings

suggest that aged animals exhibiting HPA dysfunction are exposed to higher circulating cumulative doses of GCs in the later phases of life (Bodnoff et al., 1995).

The increased exposure to the highly catabolic GCs appears to be associated with the loss of hippocampal neurons and the emergence of cognitive deficits in the aged rat. Landfield et al. (1978) and Sapolsky et al. (1984; 1985) first made the suggestion that adrenal steroids have a role in neuronal aging in the hippocampus. The led to the formulation of the glucocorticoid cascade hypothesis which states that GCs participate in a feed-forward cascade of effects on the brain and the body, in which progressive GC-induced damage to the hippocampus promotes progressive elevation of adrenal steroids and dysregulation of the HPA axis. Among a population of aged rats, the magnitude of hippocampal neuron loss and memory deficits is positively correlated with the increase in adrenal activity. In fact, adrenalectomy at mid-life (with low-level B replacement) results in reduced neuron loss in the hippocampus and improved cognitive functioning compared with intact, control animals (Landfield et al., 1981). Sapolsky et al. (1985) has found that young animals treated with exogenous B in the upper physiological range showed profound neuron loss in the hippocampus following three months of treatment. Issa et al. (1990) examined HPA function in aged rats that were tested for spatial learning impairments. They found increased basal and stress-induced HPA activity in cognitively impaired rats compared to either cognitively unimpaired or young adult controls. The presence of the cognitive impairments and concomitant increased HPA activity was associated with hippocampal neuron loss. Bodnoff et al (1995) have demonstrated significantly reduced hippocampal synaptic plasticity (dampened prime-burst potentiation) and impaired acquisition of a spatial task in animals treated with both medium-B and high-B from mid-age onward. These changes in synaptic plasticity were not accompanied by neuron loss in CA1 and CA3 pyramidal cell layer in the hippocampus. This suggests that cognitive impairments as a result of long-term exposure to GCs result in spatial learning deficits, but the impairments do not appear to be the consequence of hippocampal neuron loss. These

data are consistent with previous *in vivo* and *in vivo* studies correlating prime burst potentiation (PBB) or LTP with age-related memory deficits (Landfield and Lynch, 1977; Landfield et al., 1978, Barnes, 1979, Moore et al., 1993). These data suggest that both the number and functional plasticity of hippocampal neurons can be affected by elevations in GCs.

What are some of the mechanisms of GC-induced neuron compromise and loss? Current hypotheses suggest that prolonged exposure to these hormones reduces the ability of neurons to resist insults, increasing the rate at which neurons are damaged by toxic challenges or ordinary attrition (Porter and Landfield, 1998). GCs increase the susceptibility of certain cell types to apoptosis, possibly acting through calcium signaling pathways (Reagan and McEwen, 1997; McConkey and Orrenius, 1997). GCs have been shown to act synergistically with excitatory amino acids (EAA). A strong input of EAA into the hippocampal CA3 region derives from mossy fibres originating from granule neurons of the dentate gyrus (DG). Prolonged exposure to EAA's has been proposed to be responsible for neuronal damage or neuronal loss seen in such pathophysiological insults as ischemia, hypoglycemia and trauma (Reagan and McEwen, 1997). Treatment of rats with phenytoin, an anti-epileptic agent that inhibits EAA release and blocks their activation of T-type calcium channels, suppresses both B- and stress-induced atrophy of hippocampal CA3 pyramidal neurons (Watanabe et al., 1992). Adrenalectomy delays neuronal damage associated with ischemia and dexamethasone treatment enhances neuronal damage produced by ischemia (Koide et al., 1986). On a related front, adrenal steroids modulate the expression of NMDA receptors in the hippocampus, with chronic GC exposure leading to increased expression of NMDA receptor binding and subunit mRNA levels (Weiland et al., 1997). Elevated GC levels also increase calcium-dependent afterhyperpolarization (AHP) by acting on voltage-sensitive calcium channels (Landfield et al., 1992; Joels and De Kloet, 1994), thereby decreasing neuronal excitability. These effects are more pronounced in aged neurons, suggesting a greater sensitivity to GCs with aging. GC inhibition of glucose

utilization may contribute to increases in EAA concentrations during hypoxic-ischemic events. GCs may also decrease the transport of glucose into neurons (Sapolsky, 1994). Neurons have very limited energy storage capacity and a high metabolic rate (Seisjo, 1981) therefore a blockade on glucose uptake might compromise the viability of hippocampal neurons. GCs can inhibit glutamate uptake by glial cells (Virgin et al., 1991). Glutamate is readily eliminated from the synapse; thus elevated GCs enhance the increase in extracellular glutamate. One of the consequences of excessive glutamatergic stimulation is an increase in extracellular calcium levels through the NMDA receptor and possibly also through voltage-dependent calcium channels. Increases in extracellular calcium may activate or regulate a myriad of cellular enzymes and cascades. In relation to neuronal death occurring by apoptosis, three targets for increased calcium levels have received particular attention: the calcium-buffering capacity of mitochondria, the generation of oxygen free radicals and the activation of calcium-sensitive endonucleases (Reagan and McEwen, 1997).

HPA Activity in Aged Humans

Existing studies in humans suggest that basal cortisol levels do not change with age (West et al., 1961; Friedman et al., 1969; Sharma et al., 1989; Waltman et al., 1991). However, neuropathology in aged humans is associated with increased basal GC levels (Davis et al., 1986; De Leon et al., 1988; Dodt et al., 1991) and increased basal GCs are associated with cognitive impairments in numerous populations. Most of the effects of increased GC exposure on cognition have been studied in disorders affecting corticosteroid levels and using exogenous administration of the synthetic compound to healthy subjects.

Mental disturbances mimicking mild dementia have been described in depressed patients with hypercortisolism (Weingartner et al., 1981; Cohen et al., 1982; Wolkowitz et al., 1990) and in steroid psychosis following corticosteroid treatment (Varney et al., 1984; Wolkowitz and Rapaport, 1989). Recent studies of patient's with Cushing's disease report significant positive correlation between hippocampal formation volume and scores on verbal

memory tests and significant negative correlation between hippocampal formation volume and plasma cortisol levels (Starkman et al., 1992). The limitations in these studies are that most of the cognitive deficits associated with corticosteroids are derived from those observed during exogenous administration of synthetic GCs. Thus these studies of endogenous disorders fail to discriminate the cognitive deficits related to increased HPA activity from those due to the underlying illness itself.

Results of longitudinal studies in healthy elderly populations have revealed that aged humans show variable patterns of HPA activity later in life. Aged subjects followed over a four year period, who showed a significant increase in cortisol levels over the 4 years and had high basal cortisol levels in year 4, showed deficits on tasks measuring explicit memory as well as selective attention compared to subjects with either decreasing cortisol levels over 4 years or subjects with increasing basal cortisol but moderate current cortisol levels (Lupien et al., 1994). They also showed a hippocampus that was 14% smaller than those of age-matched controls who did not show progressive cortisol increases and were not cognitively impaired (Lupien et al., 1998). Studies report that the hippocampus is essential for a specific kind of memory, notably declarative (Cohen and Squire, 1980) or explicit memory (Graf and Schacter, 1985), selective attention and spatial memory (Lupien et al., 1998). Thus, these findings suggest that increased GC levels can influence hippocampus-dependent memory in aged humans and are related to a significant decrease in hippocampal volume and that increased GC levels are only seen in a subpopulation of humans, suggesting individual differences in the aging process. This obviously begs the question as to why some individuals show increased F with age, while others show stable F levels and still others show decreasing F levels.

Individual variation in aging: potential sources of variation.

The question of individual differences in brain and body aging involve a discussion on the possible sources of these individual differences. These include early environmental, dietary and lifestyle factors that may influence the aging process.

The Early Environment: Prenatal Stress and Postnatal Handling

In animal models unpredictable prenatal stress (PS) causes increased emotionality and increased reactivity of the HPA axis and autonomic nervous system and these effects last throughout the lifespan. Postnatal handling in rats which involves brief daily separation from the mother, counteracts the effects of prenatal stress and results in reduced emotionality and reduced reactivity of the HPA axis and autonomic nervous system (Ader et al., 1968; Ader and Grota, 1969;Hess et al., 1969; Levine et al., 1967). The vulnerability of the hippocampus to age related loss of function is in part, determined by these early environments: prenatal stress increases and postnatal handling decreases the rate of brain aging (Dellu et al., 1994; Meaney et al., 1988). Age-related decline of gonadal function reduces the beneficial and protective actions of these hormones on brain function. At the same time, age-related increases in adrenal steroid activity promote age-related changes in brain cells that can culminate in neuronal damage or cell death. Lifelong patterns of adrenocortical function determined by early experience can contribute to the rates of brain aging.

Numerous studies demonstrate that prenatal stress results in various alterations that are evident from both early and late ages (Takahashi, 1998). Pregnant animals have been subjected to: conditioned avoidance training, immobilization, restraint, suspension, crowding, repeated electric tail shocks, noise and saline injections (Weinstock, 1997). The stress is applied daily throughout gestation or during the last week only on an unpredictable basis three times weekly in rats or during the mid-late gestational period in monkeys. Data from studies using different types of gestational stress in rodents and non-human primates

support the existence of an abnormal regulation of the HPA axis in the adult and even aged offspring. Even under basal conditions, plasma B levels are higher in adult PS rats and juvenile rhesus monkeys (Clarke et al., 1994; Fride et al., 1986; Peters, 1982). The effect of prenatal stress on plasma ACTH and B is more marked in females (McCormick et al., 1995). Impairment of the feedback regulation of the HPA axis in PS rats is more readily detected after exposure to stress as indicated by greater (Fride et al., 1986; McCormick et al., 1995; Takahashi et al., 1992; Weinstock et al., 1992) or more prolonged (Henry et al., 1994) elevation in plasma B than in control rats. Intact adult PS rats that have higher circulating B levels have fewer type I and type II hippocampal GR than controls (Henry et al., 1994). A recent study by Schneider et al. (1998) found that the effects of prenatal stress persisted until 18 months of age in rhesus monkeys: prenatally stressed monkeys had higher CSF levels of MHPG and 3,4-dihydroxyphenylacetic acid than their controls when tested at 8 and 18 months of age. Prenatally stressed monkeys also spent more time clinging to their surrogates and exploring while controls showed more locomotion and social play with their cage mates. This is consistent with data from rodent studies suggesting that PS produces offspring that exhibit behavioral responses indicative of heightened emotionality or fear and anxiety. For example prenatally stressed rats in adulthood prefer the closed safe arms of the elevated plus maze (Fride and Weinstock, 1988; Vallee et al., 1997), are less active in the open field (Wackshlack and Weinstock, 1990; Vallee et al., 1997) but are not impaired on the acquisition of a spatial learning task (Vallee et al., 1997).

Most of the data on the effects of prenatal stress and the postnatal environment in humans comes from retrospective studies on children of mothers who experienced various forms of stress during pregnancy (Weinstock, 1997). The children who were followed for 2-10 years showed delays in motor development and behavioral abnormalities. These included excessive clinging, crying, hyperactivity, unsociable and inconsiderate behavior (Meier, 1985; Stott, 1973). Another study reported a higher incidence of hyperactivity-

attentional deficit disorder in children exposed to stressful events during the pre- and perinatal period (Clements, 1992). In fact, the adverse consequences of long-term loss of maternal care (a stress itself) are well documented in humans. A well described syndrome of growth arrest, failure of emotional connection with caretakers and retarded neurobehavioral development results from inadequate caretaking (Gardner, 1972; Glaser et al., 1968; Powell et al., 1967a,b). The most recent examples derive from infants raised in orphanages in Eastern Europe, who have experienced significant retardation of growth as well as neurobehavioral development (Albers et al., 1997).

Recent studies in rats suggest that prolonged maternal separation (MS, animals that are separated from their mothers for three hours per day for the first two weeks of life) results in an enhanced response to stress in adulthood, while repeated transient separation (neonatal handling) which results in enhanced maternal caretaking produces adults with diminished stress responsivity (Meaney et al., 1996; Liu et al., 1997). Neonatal handling involves separating the pups from the mother for the first 2 weeks of life, for 15 minutes per day. This manipulation in instrumental in establishing the emotionality and reactivity of the adrenocortical systems that will be stimulated later in life. It is the subsequent actions of the HPA axis in adult life that plays a major role in determining the rate of brain and body aging. In response to a wide variety of stressors, handled (H) animals, as adults, secrete less ACTH and B and show a faster return to basal B levels following the termination of stress than do non-handled (NH, animals that are left undisturbed for the first two weeks of life) and maternally-separated rats (Levine, 1967; Ader and Grota, 1969; Hess et al., 1969; Meaney et al., 1985, 1989). Young adult H and NH animals do not differ in basal B levels at any time point over the diurnal cycle (Meaney et al., 1989; 1991) but H rats have lower hypothalamic synthesis of CRH and AVP (Meaney et al., 1995). These animals also do not differ in their levels of corticosteroid-binding-globulin (CBG); this is of considerable importance since brain uptake of B appears to approximate the non-CBG bound portion of

the steroid (Partridge et al., 1983). Thus differences in total B are likely predictive of differences in brain uptake of the steroid.

The mechanism for these differences in stimulated B levels lies, in part, at the level of the hippocampus and frontal cortex. H rats show increased glucocorticoid receptor (GR) mRNA expression and binding capacity in the hippocampus and frontal cortex. In addition, GC feedback sensitivity is enhanced in the H rat: both B and dexamethasone inhibit ACTH responses to stress more effectively in H rats (Meaney et al., 1989). This increase in GR sites in the hippocampus is a critical feature of the handling effect on HPA function. Increased receptor density increases the sensitivity of the hippocampus to circulating GCs, enhancing the efficacy of negative feedback inhibition over HPA activity, and serving to reduce the post-stress secretion of ACTH and B in H animals. These differences are as late as 24-26 months of age (Meaney et al., 1988; 1991; 1992), indicating that the handling effect persists over the entire life of the animal. Throughout life, H animals have significantly higher hippocampal GR than do NH animals (Meaney et al., 1988; 1991, 1992), although both groups do show a loss of receptors with age. However, as late as 24 months of age, H rats secrete less B during restraint stress and terminate B secretion following stress than NH rats. Moreover, the age-related rise in basal B, often seen in aged rats, occurs only in the NH rats. There is a twofold increase in basal B in aged NH rats in the PM phase of the light cycle and a 40% increase during the AM phase (Meaney et al., 1992). Old NH rats also show a decrease in CBG in the PM, which is associated with a rise in free B that approximates levels achieved during stress. Thus, over the life span, cumulative exposure to GCs is greater in NH animals.

Among NH animals, but not H animals, there is a significant loss of neurons in both the CA1 and CA3 subfields of the hippocampus with age (Meaney et al., 1988; 1991). Animals screened at 6 months of age do not differ in neuron density; H attenuates the loss of hippocampal neurons at a later age. As the hippocampus has a critical role in learning

and memory, H rats, which are spared neuron loss with age, show less evidence of age-related cognitive impairments than NH rats. 24-month old NH rats take significantly longer to locate the platform than the young, 6-month old rats and 24-month old H rats perform as well as the 6-month-old animals.

This diminished rate of hippocampal neuron loss in the aging H rats most likely reflects the lower cumulative lifetime exposure to GCs. Neonatal handling reduces the HPA response to stress and this persists over the lifespan. One consequence of GC hypersecretion is accelerated neuron loss in the aging hippocampus and a consequence of hippocampal damage is reduced adrenocortical negative feedback sensitivity and GC hypersecretion. These findings suggest that in a normal population of laboratory rats, individual differences in HPA activity should serve as a predictor of age-related hippocampal pathology. In other words, if HPA dysfunction is associated with hippocampal pathology and not merely age, then a sample of aged, cognitively-impaired (AI) and aged, cognitively-unimpaired (AU) should differ considerably in HPA activity. Cognitive impairment on the Morris water maze occurs when the performance of the animal is greater than 2 standard deviations away from the mean of the young animals; unimpaired refers to a performance that was less than one half a standard deviation away from the mean of the young rats (Issa et al., 1991). According to this criteria, about 30% of the animals in a general population of laboratory rats are designated as ACI and about the same as ACU (Issa et al. 1991). Gage et al. (1984) and Gallagher and Pelleymounter (1988) have found this same extreme variation in cognitive decline in aged rats. In the Issa et al (1990) study, both groups of aged animals (impaired and unimpaired) show a loss of hippocampal neurons; the decrease in the ACI rats is substantially higher. The ACI rats show increased basal and post-stress plasma ACTH and B levels, whereas HPA activity in the ACU animals does not differ from the 6-month old rats. The loss of GR in the hippocampus is substantially greater in the ACI animals; they also show a significant decrease in hippocampal mineralocorticoid receptor (MR) density and decreased MR mRNA. Taken

together, these data on neonatal handling strongly suggest that increased GC levels are selectively associated with the occurrence of hippocampal pathology and impaired cognition later in life. Therefore prenatal stress and postnatal handling are two useful models for studying individual differences in stress-induced reactivity that persist into old age.

Diet/Body Mass

While manipulating the early environment can have profound, long-lasting and even permanent effects on the aging process, differences in the dietary lifestyle across the lifespan of the individual plays a critical role as well. For many years, insulin insensitivity and glucose tolerance have been considered necessary correlates of the aging process, as a number of studies reported that there is a remarkable increase in the mean 2 hour blood sugar levels with advancing age (Davidson, 1979; Chen et al., 1985; Reaven et al., 1989). However many studies have found that much of the observed carbohydrate intolerance of older people may be caused by factors other than biological aging per se, and that dietary and exercise modifications may substantially blunt the emergence of carbohydrate intolerance and insulin resistance with increasing age (Rowe and Kahn, 1987). Zavaroni et al. (1986) evaluated the relative contributions of obesity, physical activity and family history of diabetes to age-related increases in glucose and insulin levels after an oral glucose tolerance test in factory workers aged 22 to 73 years. The initially strong correlation between age and both post-prandial glucose and insulin levels became significantly weaker when the effects of exercise and diet were taken into account. Barnard's group (Barnard et al., 1995) found that the diet not aging was responsible for causing skeletal insulin resistance in aged rats. No significant differences were observed between 6- and 24-month old rats raised on a low fat, high carbohydrate diet on serum glucose and insulin or for basal or insulin-stimulated glucose transport, providing strong support that aging per se does not lead to insulin resistance. When 24 month old animals raised on the low fat, high carbohydrate diet were compared to rats raised on a high fat diet major, differences were

observed: fasting serum insulin was significantly higher in the high-fat diet group and insulin-stimulated glucose transport was significantly reduced (Barnard et al., 1995). High-fat diets not only affect metabolic indices of aging; a number of studies suggest that high fat diets can cause cognitive impairments in adult rats (Greenwood and Winocur, 1990;1996; Winocur and Greenwood, 1993; 1999) and that it is saturated fats that are responsible for the impairment (Greenwood and Winocur, 1996). While these authors did not measure GC levels in these animals, it is conceivable that long-term fat feeding increases plasma corticosterone and may, in turn, render hippocampal neurons vulnerable to GC-induced damage. The authors (Greenwood and Winocur, 1996) did find changes in brain fatty acid profiles, which were associated with alterations in brain membrane composition.

While diet is a critical predictor for the development of glucose insensitivity in the rat, body composition seems to play a crucial role as well. Barzilai and Rossetti (1996) found that in aged animals with increased body weight, fat mass and epidydymal fat, postabsorptive insulin and FFA levels increase concomitantly. Thus, there is an age-related increase in fat mass and two of the consequences of this are increased FFA and decreased insulin sensitivity. Machado et al. (1991) found that lean (20% reduction in body weight caused by caloric restriction) 12 month old rats submitted to a glucose tolerance test, have glycemic and insulinemic profiles that are similar to those observed in 2 month old animals. In obese rats (those allowed ad lib access to feed), however, insulin secretion is increased and glucose tolerance decreased suggesting again that obesity is the major cause of altered insulin secretion in aged rats.

The finding that high amounts of fat in the diet or increased body fat may increase the risk of developing insulin resistance has been thoroughly investigated. The pioneering work by Himsworth (1935) demonstrated that a high fat diet decreases glucose tolerance and impairs insulin sensitivity. A substantial amount of *in vitro* work has accumulated showing a reduced insulin-mediated glucose metabolism in adipose tissue (Susini and

Lavau, 1978; Olefsky, 1978) and muscle (Grundleger and Thenen, 1981) from animals fed diets high in fat or simple sugars. Ip et al. (1976) demonstrated that fat cells (adipocytes) isolated from rats that were on high fat diet (lard) for 5-7 days showed a decreased response to insulin and bound less insulin when compared to cells isolated from rats fed a high glucose diet. Sun et al. (1977) extended these findings to include decreased binding capacity of insulin to its receptor in the liver following exposure to a high-fat diet. *In vivo* work has extended these findings: Storlien et al. (1986) and Kraegen et al. (1986) found that consumption of diets that were high in fat (polyunsaturated fat) led to major impairments in insulin action. Only 3-4 weeks on the diet were necessary to see reductions of up to 50% in insulin-stimulated glucose metabolism with insulin levels raised to the physiological range. In fact dietary fat can prolong the insulin resistance that is normally seen from day 1-21 in newborn rats. Rats fed normal laboratory chow following weaning show normal insulin sensitivity while rats fed a high fat diet show continued insulin resistance (Issad et al., 1988).

Body mass index (BMI) changes can also lead to impairments in insulin secretion and glycemic control. Prospective studies in both men and women suggest that the BMI and waist/hip circumference ratio (WHR) are the most powerful predictor of NIDDM (Bjorntorp, 1992). Obesity is also a risk factor and in particular, abdominally localized obesity is strongly associated with increased risk of developing NIDDM. A study carried out in a population with an extremely high prevalence of obesity and NIDDM, the Pima Indians investigated the effect of duration of obesity on the development of diabetes. It was found that the longer the duration of obesity, the higher the number of incident cases of diabetes (Everhart et al., 1992). A study by Perry et al. (1995) showed a remarkable exponential increase in the risk of diabetes as BMI increased, even over a range of BMI not considered to be obese. In both men and women, a high WHR which is an index of visceral obesity is associated with a high risk of cardiovascular disease (Bjorntorp, 1992). Visceral obesity produces metabolic and circulatory changes through an increased flow of free fatty

acids (FFA) to the liver, leading to the development of insulin resistance and increased lipoprotein synthesis, resulting in glucose intolerance, hypertension and hyperlipidemia. There are marked changes in lipoproteins: both the size and number of vLDL particles increase in obesity. The number of LDL particles also rises and lipoprotein lipase activity decreases resulting in decreased HDL (Depres, 1994).

High fat diets have similar effects. Lipid availability can inhibit *in vivo glucose* utilization in skeletal muscle in rats and man (Jenkins et al., 1988; Nuutila et al., 1992). Skeletal muscle insulin action deteriorates under conditions when triglyceride levels are high (Storlein et al., 1997). In contrast insulin action is improved by lowering circulating triglycerides with pharmaceutical agents (Storlein et al., 1993) or with weight loss/exercise (reviewed in Brownwell, 1998). In addition to alterations in glucose/insulin dynamics, the consumption of high-fat diets and android/gynoid obesity are both associated with increased levels of GCs. This is of relevance since GCs inhibit the antilipolytic, or fat-releasing effect of insulin, by inhibiting the enzyme hormone-sensitive lipase (Peeke and Chrousos, 1995). In addition fat storage is enhanced by an increase in the activity of the fat storage enzyme, lipoprotein lipase, the activity of which is stimulated by prolonged exposure to high levels of both cortisol and insulin. The net result is an ever-increasing accumulation of fat, primarily in the visceral depot, where increased GR density, enlarged adipocytes, increased cortisol and insulin and decreased lipolysis cause energy storage (Fain et al., 1971). The terms "metabolic syndrome" or "Syndrome X" were used to describe this cluster of metabolic diseases seen in conjunction with obesity including hypertension, hyperlipidemia, hyperinsulinemia, and insulin resistance (Reaven, 1988).

Other models indicating that a nutritional change in lifestyle might favorably affect the lifespan are caloric restriction. Under the usual experimental paradigms, calorically restricted animals receive a balanced reduction of the protein, carbohydrate and fat content of the diet without a reduction in micronutrients. Typically, caloric restriction is 30-50% of

ad libitum consumption. Calorically restricted rats and mice show a decrease in the incidence and proliferative rate of spontaneous and chemically induced neoplasia, they demonstrate a significant increase in life-span, lower blood glucose and higher B. Harris' group (1994) looked at how caloric restriction can affect basal glucose as well as the response to glucose challenge in mice across the lifespan. In general they found that glucose does not change as a function of age but does change as a function of energy restriction. 50% Caloric restriction lowered serum glucose significantly and overall responses to glucose challenge were unaffected by both age and caloric restriction (Harris et al., 1994). Masoro et al. (1992) found that dietary restricted rats maintained mean 24-hour plasma glucose and insulin concentrations 15% and 50%, respectively, below those of *ad libitum* fed rats. Caloric restriction also causes chronic hypercorticism; this nutritional "stress response" differs from other stress situations or GC therapy (Frame et al., 1998). B levels are increased above those of their *ad libitum* controls only during a limited circadian period prior to and coincident with feeding activity. In the study by Harris et al. (1994) average serum B concentrations were elevated at 7 months by restriction, especially at night and long after feeding but there were no overall differences with age or diet in average B concentrations. Ma et al. (1989) found similar results in their study with energy-restricted rats. This type of intermittent elevation in B appears to be less damaging to mitogenic processes than continuously elevated B levels (Hart et al., 1999). Because the elevation in B is not associated with chronic hyperglycemia and hyperinsulinemia, insulin resistance as a result of GC-induced hyperglycemia does not occur. Finally significant elevations in B in response to caloric restriction only occurs during the early stages of restricted feeding (Stewart et al., 1988).

A number of groups have also examined the effects of malnourishment and protein restriction on HPA axis functioning, hippocampal morphology and indices of carbohydrate metabolism. Andrade et al. (1995) recently found that feeding adult rats a low (4%) protein diet for 18 months significantly reduced the total number of hippocampal neurons and that

even switching from a low to a high protein diet does not preserve the neurons. There is substantial evidence suggesting that switching to a low protein diet is a stress (Garcia-Belenguer et al., 1993); as a result plasma levels of GCs increase, plasma GH levels decrease (Harel and Tannenbaum, 1993) resulting in a state which can induce marked hippocampal neuronal damage (Sapolsky et al., 1986; Gould et al., 1992).

Environmental Enrichment

The effects of manipulating the housing and rearing environment of rats have been studied extensively. Hebb (1949) was one of the first to report that animals reared in a "free environment" performed better in learning tasks than rats from a "restricted environment." These were followed by studies by Rosenzweig (1979) who set up the enriched versus impoverished environmental paradigms. Environmentally enriched (EE) rats are housed in large cages that contain ladders, toys and tunnels, and are changed on a weekly basis. Juvenile and young adult rats housed in enriched environments undergo a series of neurochemical, neuroanatomical and behavioral changes. These include increased dendritic branching, increased number of synapses per neuron and synaptic size (Turner et al., 1983; Volkmar et al., 1972; West et al., 1972). These changes occur primarily in the hippocampus and visual cortex, in addition to the cerebellum. Enriched animals also display less emotionality scores than isolates in the open field and demonstrate a greater diversity of exploratory behavior (Fernandez-Treul et al., 1997). More recent work in this field has demonstrated a link between increased hippocampal nerve growth factor (NGF) levels and improved performance on the Morris Water Maze (Mohammed et al., 1990; 1993; Henriksson et al., 1992) in response to brief periods of EE. Olsson's group (1994) has shown that environmental enrichment induces glucocorticoid receptor (GR) expression in specific hippocampal subfields (CA1 and CA2) and an increased expression of mRNA encoding the NGF-induced immediate early gene NGFI-A in CA2.

In addition to the effects of EE on HPA status, a number of studies have focused on how other systems may be affected by this manipulation. Park et al (1992) has shown that enrichment causes a long-lasting increase in caudate acetylcholine (ACh) synthesis and it also primes the cortex and hippocampus to a training experience with increased ACh synthesis. Klein's group (1994) has shown that environmental enrichment lowers anxiety in the presence of a predator stress. In a similar study, Haemisch's group (1994) showed that mice in the enriched condition attacked intruders significantly more frequently than mice that were group housed in standard laboratory cages. The position of the dominant male was less stable in enriched conditions and plasma corticosterone titers were significantly elevated in dominant mice in the enriched condition. Conversely, environmentally impoverished (EI) rats housed individually in single, opaque cages show behavioral and physiological alterations. A number of groups have demonstrated that social isolation can cause deficits on the acquisition of spatial tasks and can produce increased startle responses (Domeney and Feldon, 1998).

The majority of studies examining the effects of EE have imposed the manipulation for a brief period (usually 30 days) during the immediate post-weaning phase. A number of studies have examined the effects of EE on the aging process, in terms of synaptic plasticity, endocrine status and behavioral parameters. EE modifies the sleep-wake pattern of aged rats (Mirmiran et al., 1986; Van Gool and Mirmiran, 1986). Similarly, the learning performance in aged rats that had spent a year in EE was improved when they were tested at 2 years of age (Doty, 1972). Rats housed in EE conditions for 509 days had higher brain weights, longer cerebral length and made significantly fewer errors in the Hebb-Williams maze than those reared in isolation (Cummins et al., 1973). Aged rats that were 766 days old and exposed to EE conditions until they were 904 days old developed thicker cerebral cortices (Diamond et al., 1985). Greenough's group (1986) has shown that EE can cause significant changes in the dendritic field of the cerebellum of EE-housed aged rats. Escorihuela et al (1995) investigated the effects of a 6-month period of EE (which began 3

weeks after weaning) on spatial learning in 24-month old hypo- and hyperemotional and rats. EE during adolescence and early adulthood prevented later age-associated learning deficits in both rat lines.

Exercise

The beneficial effects of physical exercise are well documented. There have been a number of studies that have demonstrated improvements in glucose tolerance and insulin resistance following exercise regimens, in older people (Seals et al., 1984; Zavaroni et al., 1986; Ivy, 1997). Low insulin secretion to glucose stimulus has been observed in pancreatic islets from exercised rats when compared with sedentary animals (Brandy et al., 1977; Zawalich et al., 1982). Endurance exercise increases the rate of utilization of all metabolic fuels, thereby decreasing glycogen content of both the liver and muscles. In addition, endurance training markedly increases insulin sensitivity, so that very much lower concentrations of insulin are required to control blood glucose (Bjorntorp et al., 1977). Seals et al. (1984) found that master athletes who averaged 60 $\pm$ 2 years of age were as lean as young untrained individuals. They also had plasma glucose responses to an oral glucose challenge that were similar to those of young healthy men and significantly better than healthy untrained men of a similar age. Numerous mechanisms of action have been proposed: exercise training results in a preferential loss of fat from the central regions of the body and should therefore contribute significantly in preventing or alleviating insulin resistance related to excess body fat (Ivy, 1997). Further, a reduction in adipocyte size via exercise training should help alleviate excessive FFA in the plasma; obese individuals are resistant to insulin suppression of FFA levels. In terms of HPA activity, chronic exercise has been shown to both increase and decrease levels of ACTH and B (Wittert et al., 1996). Despite the possibility of sustained activation of the HPA axis following exercise, studies examining the effects of exercise on cognition in aged individuals and rats suggest that exercise can be protective against age-associated decline in cognitive performance. In fact a

number of studies have provided morphological evidence suggesting that physical activity increases BDNF and NGF mRNA in the hippocampus (Neeper et al., 1996), which have been shown to be deficient in the aged brain (Larkfors et al., 1987; Larkfors et al., 1988) and that physical activity may induce increases in local cerebral glucose utilization (Vissing et al., 1996).

In sum, these studies suggest that aging itself is not a uniform process. There are marked individual differences in both the course and manner in which an individual ages and there are numerous sources for this variation, including cumulative exposure to GCs over the lifespan, dietary variables, exercise and the quality of the prenatal, early and even mid-life environments.

STATEMENT OF THE PROBLEM

The HPA axis is a highly integrated process involving both central and peripheral systems. It plays an important role in many functions, including feeding, sexual behavior, cognition, emotion and of course the response to stress. The synthesis, release and containment of glucocorticoids are regulated by numerous factors; these factors receive reciprocal regulation by the axis. The development of the stress response itself is highly sensitive in the early neonatal period, such that subtle manipulations in the early environment contribute to a permanent change in the development of both behavioral and neural systems associated with the HPA axis. Furthermore, the axis is involved in the day to day metabolic status of an organism: changes in both the quality and quantity of the diet are associated with a shift in the rhythm of the axis, as the axis is finely tuned to the daily feeding cycle. Perturbations to the axis itself, such as under conditions of extreme stress or illness are associated with the development of metabolic disorders and changes in dietary preferences across the cycle. The axis is also relatively plastic later in life such that changes in the environment, such as dietary changes, environmental enrichment or social isolation, even as late as mid-life in the rat, can have permanent and profound effects on the amount of GC-exposure that the organism is subject to. In addition to all of the environmental factors, there is a large amount of individual variability in the aging process. One issue that has been at the core of these studies is the question of why some individuals age successfully while others do not (increase in B, cognitive impairments, obesity and related metabolic dysfunction)?

The present studies have attempted to address the following issues: do manipulations in the early neonatal environment known to affect the both development of the HPA and metabolic axes have effects on later food preferences and indices of carbohydrate metabolism? Do perturbations to the HPA axis, such as during chronic stress, affect food choices and do these food choices themselves then further modulate the

production and release of F and the psychological response to stress? Do particular macronutrients, such as fat, have direct affects on basal and stress-induced HPA and glucoregulatory function and if so, can a high-fat diet accelerate the aging process? Finally, are there environmental manipulations, through their effects on circulating GCs, that can be introduced late in life to prevent some of the adverse effects of central nervous system aging?

THE PRESENT STUDIES

Therefore the present studies have attempted to investigate the reciprocal relationship between HPA axis function and environmental/dietary variables, introduced at numerous points across the lifespan in young and aged rodent and human populations.

Study 1 The Early Environment and the Development of Individual Differences in Basal and Stress-Induced Food Intake and Body Weight

The prenatal and postnatal environments exert long-term influences on emotional and physical health. Early life events have profound effects during what is termed the "critical period" of development. Infants given adequate food, water and warmth, but who are deprived of being touched and held, show retarded emotional and physical development (Bowlby, 1968). Premature infants who receive additional tactile stimulation to compensate for weeks of life in an incubator grow faster and gain more weight than infants who do not receive such stimulation (Field and Schanberg, 1990). Chronically abused and neglected children tend to be slower to talk, underweight, less able to concentrate and fall behind in school compared to well-cared for children (Vondra et al., 1990; Hanson et al., 1989).

The results of animal studies suggest that these early effects persist throughout life. Experiments performed in the late 1950s and 1960s revealed that brief periods of separation between mother dams and their pups decrease the offsprings' corticosterone response to stress and attenuate fearfulness in novel environments (Ader and Grota, 1969; Hess et al., 1969; Levine, 1957; Levine, 1961; Levine et al., 1967). Meaney's laboratory has found that both brief and longer periods of maternal separation (termed "handling or H, and maternal separation or MS", respectively) can alter the development of specific biochemical systems in the brain and lead to stable individual differences in biological responsiveness to stress (Meaney at al., 1994). Handled rats (separated from their mothers for 15 minutes per day for the first 21 days of life) display a decreased HPA response to stress, increased GC receptor binding density in the hippocampus, increased negative feedback efficacy, and lower hypothalamic CRF mRNA and content (compared to undisturbed, non-handled (NH) rats) (Meaney et al., 1995). More prolonged maternal separation has quite different effects. MS rats, separated for 180 minutes per day for the first 21 days of life, have increased levels

of CRF in the hypothalamus, decreased GR binding and reduced negative feedback efficacy (Plotsky and Meaney, 1993).

In addition to the effects of postnatal manipulations on the HPA axis, a number of groups have examined both the short and long-term influence of postnatal manipulations on metabolic parameters. Vallee et al. (1996) found that prenatally stressed (PS) rats produced offspring showing evidence for hyperglycemia, while H animals had higher body weights and food intake compared to both the PS and control, NH rats. McIntosh et al. (1999) found that H rats displayed less anxiety on the elevated plus maze compared to NH rats, and that MS rats weighed less than both H and NH rats. These findings suggest that early environmental modulation of HPA axis activity may be a potent contributor to the development of anxiety or eating disorders.

A number of recent human studies have examined the relationship between the early rearing environment and the development of specific disorders in childhood, adolescence and adulthood. In particular, numerous studies have attempted to clarify how the family background and early postnatal environment are possibly linked with the development of eating disorders. The family environment of bulimics for example has been described as lacking in expressive communication and lower in perceived care and warmth (Calam et al., 1990). Kinzl's group (1994; 1997) has found that an adverse family background including, but not limited to, physical and sexual abuse and neglect may be important etiological factors in the development of anorexia nervosa. Interestingly, alterations in HPA axis function (including elevations in urinary and plasma F) have been found in individuals with anorexia nervosa and bulimia.

We therefore examined how these neonatal manipulations might affect short and long-term weight gain, food intake, food preferences and insulin dynamics. Since body weight and food intake are dependent on the activity of the HPA axis and alterations in HPA activity are found in numerous eating disorders, it would follow that early life manipulations that affect later HPA activity might also affect eating patterns and indices of carbohydrate

metabolism. Further, since these early environmental manipulations do not seem to affect basal HPA activity (Meaney et al., 1995), we decided to also explore body weight changes and food intake before, during and following both an acute and 21-day chronic stressor.

Methods

Subjects

The animals used in these studies were the male and to a lesser extent, the female offspring of pregnant Long-Evans, hooded rat dams (Charles River, St. Constant, Quebec). Handling consisted of removing the mother and then the pups from their home cage and then placing the pups in a cage lined with bedding material. Pups, and then the mother were returned to their home cage 15 minutes later. Handling occurred once a day beginning the day after birth for the first 21 days of life. Maternal separation involved the same procedure; however, the pups remained away from the dam for 180 minutes. The non-handled (NH) animals were left completely undisturbed for this period of time; all animals received cage maintenance only on day 14. Weaning occurred at 21 days of age and rats were housed in same-sex, same-treatment groups of 2 animals per cage. Animals were tested at both 3 and 9 months of age. During day 1-21 all dams and their litters were provided with ad lib food (Purina Lab Chow) and water and housed in a temperature- and humidity-controlled environment. Animals were maintained on a 12:12 light: dark cycle with lights on at 08:00h and off at 20:00h. All experimental procedures were conducted in accordance with Canadian Council on Animal Care guidelines.

Body Weights and Food Intake

Body weight (in grams) was measured every second day for all H and MS rats starting from postnatal day 5 until postnatal day 21 (n=45 per group). A subset of male H and MS (n=20 per group) rats were consistently weighed from postnatal day 21 until the termination of each study. In order to ensure that NH rats were left completely undisturbed

during day 1-21, body weights for all male NH rats were taken every second day starting from postnatal day 21 (n=20). From day 40 until day 68 a subset of 10 females from each treatment group was weighed.

Food intake was measured every second day from postnatal day 45 until postnatal day 85 in a subset of 16 male rats from all treatment groups. Lab chow was placed in a food dispenser that was attached to the side of the cage. Food dispensers were designed to avoid spillage and intake was measured by weighing the amount of food placed in the feeders and subtracting the remaining uneaten amount. At nine months of age, male rats (n=20 per treatment group) were once again evaluated on regular daily chow intake for 12 days and body weights were taken once per week before, during and following the chronic stress. As rats are social animals, we housed 2 per cage and the amount of total chow consumed per cage was divided by two to get an index of consumption per rat.

Baseline Macronutrient Selection

A subset of 3 month old (n=16 per group) and later on in the study, 9 month old-males (n=7 per group) were chosen for the macronutrient selection studies. Animals were housed 2 per cage and maintained on a free-feeding self-selection paradigm with three pure pre-weighed macronutrient diets of protein, carbohydrate and fat and water available ad libitum. The protein diet (caloric density of 3.7 Kcal/g) consisted of 93% casein (granulated enzymatic casein, ICN Pharmaceuticals), mixed with 4% minerals (Salt Mixture Briggs, ICN), 2.97% vitamins (Vitamin Diet Fortification Mixture, ICN) and 0.03% cysteine (*L*-cysteine hydrochloride, ICN). The carbohydrate diet (3.7 Kcal/g) consisted of 28% dextrin (ICN), 28% cornstarch (ICN) and 37% sucrose (ICN) mixed with 4% minerals and 3% vitamins. The fat diet (7.7 Kcal/g) consisted of 86% lard (ICN), mixed with 8% minerals and 6% vitamins.

The three macronutrients were provided simultaneously in separate food dispensers designed to avoid spillage and braced to the side of large home cages. The placement of the

hoppers was changed every time food intake was measured, to ensure that there were no positional preferences. At the time food intake was measured, rats received a fresh replacement of food. Baseline macronutrient selection (termed "pre") was measured for 7 days and weighed every second day.

Acute and Chronic Stress-induced Macronutrient Selection

Three-month-old rats were exposed to the acute stress. Following one week of baseline macronutrient selection, rats were exposed to a single episode of acute stress in the morning. The stress was a resistance to capture stress, where each rat was placed in a novel open field for 5 minutes, and was then retrieved by an experimenter wearing a gloved hand (resistance to capture ratings and data are not included in this thesis). Rats were then returned to the home cages and their macronutrient selection was measured on the day of the stress (termed "stress") and then measured every two days for 7 days following the acute stress (termed "post"). Rats were then maintained on regular lab chow until they reached nine months of age.

Macronutrient selection before, during and following a 21-day period of variable chronic stress was assessed in 9 month old rats (n=11 per group). Rats underwent an assessment of their preferences during 21 days of differing stressors (described below). Macronutrient choice was also assessed for one week following the end of the chronic stress period. Rats were then returned to regular chow for one month until the end of the study.

Unpredictable Variable Stressor Paradigm

Every day at approximately the same time (10:00-13:00) rats received a stressor. The stressor lasted between 15 minutes and three hours, depending on the type of stressor used. Rats did not receive the same stress on two consecutive days; we chose this kind of chronic stress to ensure that rats did not adapt to the stress. The types of stressors used

included 3-hour restraint, 3-hour exposure to cat feces, 3 hours of cold, 15 minutes of swim stress and 15 minutes of tail pinch.

Glucose Tolerance Test and Insulin Radioimmunoassay

One month after the termination of the chronic stressor regimen, rats were assessed on a glucose tolerance test. After a 12-hour food deprivation, a blood sample (200 μl) was taken from the tail vein rats at 08:00 h to assess basal plasma insulin levels. This was termed the "pre" sample. At 10:00 am, rats were injected i.p, with 2.5 g/kg 50% glucose and blood samples were taken at 15, 30, 45, 60, 75 and 120 minutes following the injection. Rats were then returned to their home cage. Plasma insulin levels were measured with a commercial kit using ^{1}125-insulin as tracer and guinea pig anti-procine serum as antibody (ICN Biomedicals). 50 μl of plasma were assayed in duplicate.

Statistical Analyses

Data were analyzed with factorial and repeated measures analyses of variance and by paired and unpaired Student's t-tests. Scheffe post-hoc tests were performed when appropriate. $P<0.05$ was considered significant.

Results

Body weights (days 1-86)

Body weights taken from male and females rats (combined) from post-natal day 5 until day 21 are shown in figure 1. In general, H rats weighed more than MS rats during this period and significantly more ($p<0.05$) on days 5, 7,9,11, 17 and 19. When only males were weighed on postnatal days 22-42, both H and NH males weighed significantly ($p<0.05$) more than MS males on days 23 and H rats weighed more than MS rats on day 24; however there were no significant differences beyond day 24 and up until and including

day 42 (Figure 2). When rats were divided by gender, males (top panel of figure 3) generally weighed more than females (bottom panel of figure 3). Male NH rats weighed significantly ($p<0.05$) more than male MS rats on day 48 of life; on day 60 male H rats weighed significantly more than male MS rats ($p<0.05$). A similar trend was found with females (bottom panel figure 3): female H rats weighed significantly more than MS females on days 48,60 and 68 while female NH rats weighed significantly more than female MS rats on day 68 only.

Baseline Chow and Macronutrient Intake

Daily average food intake per rat of regular rat chow is shown in figure 4. In general H ate more than both NH and MS rats. Specifically, on days, 55, 75, 80, and 85, H rats ate significantly more than both NH and MS rats and ate significantly more than MS rats on day 45 as well. We then examined the pre-stress consumption of protein, fat and carbohydrate and found that H, NH and MS rats generally consume similar amounts of protein but that H and NH rats consume significantly ($p<0.05$) more fat when compared to MS rats and NH rats consume significantly more carbohydrate when compared to both H and MS rats (figure 5). All rats consumed most of their daily intake in the form of protein, with the remainder of their consumption coming from fat and carbohydrate.

Acute Stress-induced changes in macronutrient intake

When we exposed rats to an acute stress of a 1-hour resistance to capture test, rats generally consumed less protein on both the day of the stress and for one week following the acute stress when compared to their pre-stress protein consumption (figure 6). H rats consumed significantly less protein on the day of stress and following stress when compared to their baseline protein consumption ($p<0.05$) but there was no significant difference in H rats' protein consumption during stress when compared to their protein consumption post-stress. NH rats consumed significantly less protein during the acute

stress when compared to their baseline consumption; however there were no significant differences in protein consumption between baseline and post-stress phases and stress and post-stress phases in NH rats. Finally, MS rats consumed significantly more protein during baseline period when compared to their consumption during stress and during the period following stress. Protein consumption was similar during all periods when we compared H, NH and MS rats to each other.

Carbohydrate consumption prior to, in response to and following an acute stress is shown in figure 7. As shown in figure 5, at baseline both H and MS rats consume significantly less carbohydrate when compared to NH rats. An acute period of stress has a significant effect on carbohydrate consumption in MS rats only. Specifically, H rats consume significantly more carbohydrate during the pre-stress and stress periods when compared to their consumption during the post-stress period but do not increase carbohydrate in response to stress. NH show the same profile with significantly greater carbohydrate consumption during both the pre-stress and acute stress period then the post-stress period. In terms of carbohydrate consumption, MS rats consume the most during stress, followed by their carbohydrate consumption during the baseline period and their lowest consumption of carbohydrate is during the period following the acute stressor.

Fat consumption is shown in figure 8. Stress does not have any significant effect on fat consumption in any group of animals. There is, however, a significant difference in fat consumption at each time point. At all three time points, MS rats consume significantly less fat when compared to both H and NH rats. H and NH rats consume similar amounts of fat at each time point. We summarized all these results by depicting the total (protein+carbohydrate+fat) consumption in figure 9. H, NH and MS rats consume similar amounts of total food at each time point. However in general, H rats consume significantly more during baseline when compared to both stress and recovery, NH consume significantly more during the baseline period when compared to their consumption during

the recovery period and finally, MS rats consume significantly more total food during the baseline period when compared to the stress and recovery periods.

The percent (%) intake of total grams consumed of each macronutrient by H, NH and MS rats at the different time points (before, during and following acute stress) are summarized in table one. The results are similar to the gram consumption represented in figures 5-9.

Baseline Chow and Macronutrient Consumption at 9 months of age

Figure 10 shows the average daily chow consumption per rat in rats aged 9 months, measured over 12 days. While individual animals vary the amount of daily food they consume over a baseline (pre-stress) period of 12 days, H, NH and MS rats as a group generally consume similar daily amounts of chow. When we gave animals a choice, NH animals consumed significantly more carbohydrate when compared to both H and MS rats and MS rats consumed significantly more carbohydrate when compared to H rats (figure 11). H rats consumed significantly more protein and fat when compared to MS rats. H and NH rats consumed similar amounts of protein and fat (figure 11).

Chronic Stress-induced changes in macronutrient intake

Chronic stress had significant effects on carbohydrate consumption (figure 12) in NH and MS rats. NH rats significantly ($p<0.05$) reduced their carbohydrate consumption during chronic stress but resumed pre-stress levels of carbohydrate following the termination of the chronic stress. MS rats consumed significantly more carbohydrate during the pre-stress period when compared to their consumption during chronic stress. H rats consumed similar amounts of carbohydrate during all three periods. Again, NH rats ate significantly more carbohydrate during the pre-stress period when compared to both MS and H rats and MS rats consumed significantly more carbohydrate than H rats during this

period. During both chronic stress and post-stress, MS and NH rats consumed more carbohydrate than H rats, while NH and MS consumed similar amounts.

Chronic stress also had significant effects on fat consumption (figure 13). H and NH rats both consumed significantly less fat during stress and post-stress when compared to their respective consumption before stress. MS rats consumed significantly less fat during post-stress than during pre-stress. H rats consumed significantly more fat when compared to MS rats during the pre-stress period only.

Stress-induced protein consumption followed a similar profile to fat consumption (figure 14). H rats consumed significantly less protein during chronic stress and following stress when compared to their consumption during pre-stress. NH rats significantly diminished their consumption of protein in response to stress but not following stress. MS rats consumed significantly less protein during both stress and following stress when compared to their baseline consumption. H rats consumed significantly more protein during the pre-stress period when compared to MS rats during that period.

Finally, total consumption is summarized in figure 15. Total consumption was diminished in response to stress in H, NH and MS rats. Furthermore, consumption remained diminished during the post-stress period in all rats. Total consumption did not differ between stress and post-stress periods. NH rats consumed significantly more total macronutrient during chronic stress when compared to MS and H rats and all rats consumed similar amounts during the post-stress period.

In figure 16, we combined all groups of animals and show the overall consumption of macronutrients before, during and following acute and chronic stress. The top and bottom panels of figure 16 depict carbohydrate, protein and fat consumption (H, NH and MS consumption combined) before, during and following acute and chronic stress respectively. Prior to acute stress (figure 16, top) 3 month old rats consume significantly more protein compared to both fat and carbohydrate; on the day of acute stress, rats generally consume significantly more carbohydrate than both protein and fat and post acute

stress, significantly more protein is consumed compared to carbohydrate and fat. Carbohydrate consumption is significantly reduced during post-stress when compared to carbohydrate consumption both before and on the day of acute stress. Protein consumption is significantly elevated prior to acute stress and while it goes down significantly on the day of stress, protein consumption increases during post stress (but is still significantly reduced compared to consumption during pre-stress). Finally fat consumption stays the same throughout all three periods.

At nine months of age (figure 16, bottom panel) and prior to the onset of the chronic stressor, rats consume significantly more protein and fat when compared to carbohydrate. In fact rats consume significantly less carbohydrate compared to protein and fat prior to, during and following chronic stress. During chronic stress there is a significant decrease in protein and fat, while carbohydrate consumption remains the same. Following stress, protein and fat consumption remain significantly reduced compared to their consumption prior to stress but do not decrease any further during the post-stress period.

The percent (%) intake of total grams consumed of each macronutrient by H, NH and Ms rats at the different time points (before, during and following chronic stress) are summarized in table 2. The results are similar to the gram consumption represented in figures 11-15 with the exception that while the total grams of protein consumed diminished in response to chronic stress in all rats (figure 14), the percent of total grams consumed from protein remained constant across all time points and within groups of animals (table 2). NH rats consumed significantly less ($p<0.05$) % of total from protein compared to MS and H rats before chronic stress and H rats consumed more ($p<0.05$) % total from protein compared to NH rats during the chronic stress period.

Body Weights and Acute and Chronic Stress

Figure 17 shows body weights before, during and following acute (figure 17, top panel) and chronic stress (figure 17, bottom panel). Prior to the acute stress H rats weigh

significantly more ($p<0.05$) than MS rats (figure 17 top). All rats weigh significantly more following the acute stress compared to what they weighted the week before, but H and NH both weigh significantly more than MS rats at this time. Prior to the 21 days of chronic stress, H and NH rats weighed more than MS rats (figure 17 bottom), but this was not significant. During the chronic stress period, H rats weighed more than MS rats and on the final day of weighing, H rats weighed more than both NH and MS rats, but only significantly more than MS rats. Only H rats showed weight gain; NH rats lost a small amount of weight following chronic stress, while MS rats maintained the same weight throughout all three periods.

Glucose tolerance test

Blood insulin levels prior to, and at numerous time following a glucose tolerance test are shown in figure 18. Basal levels ("pre") of insulin were significantly higher in MS rats when compared to both H and NH rats. Insulin levels were elevated following glucose injection (time "15") in H and NH rats but MS rats showed virtually no change in insulin levels in response to the injection of glucose. Insulin levels were similar at all time points following glucose injection; however while insulin levels declined after the initial rise in H and NH rats, MS rats insulin rose at 90 and 120 minutes following glucose injection.

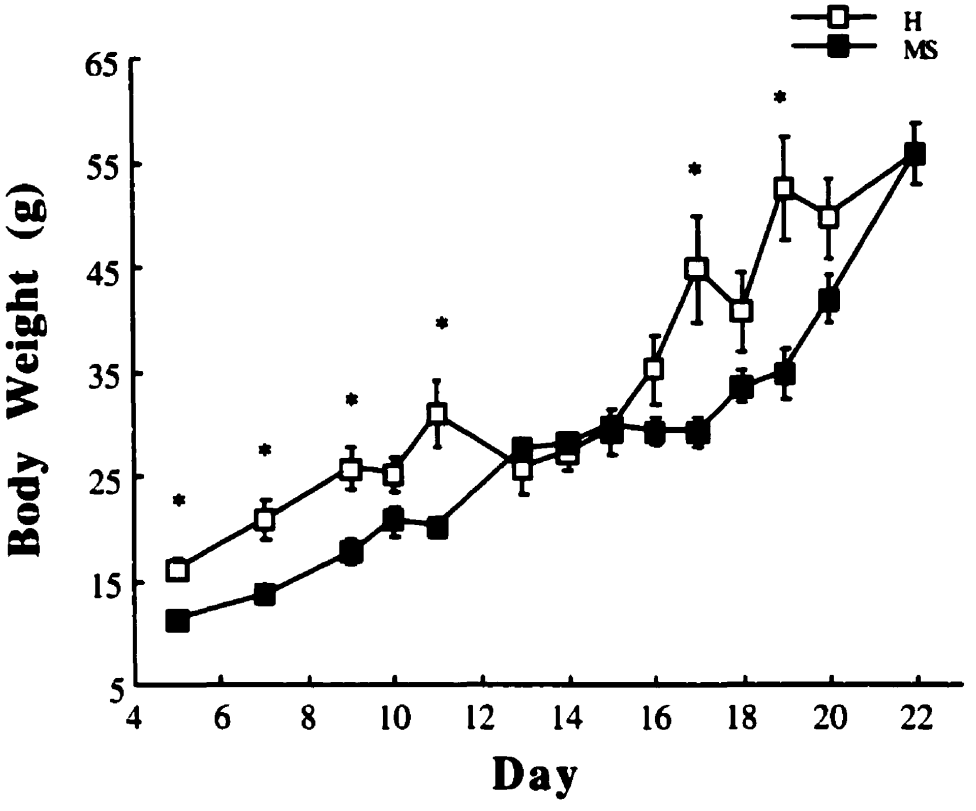

Figure 1. Mean (± SEM) body weights taken for the first 21 days of life in animals that were separated for 15 minutes (H, n=45) or 180 minutes (MS, n=45) per day from their mother from day 1-14. * H significantly different from MS.

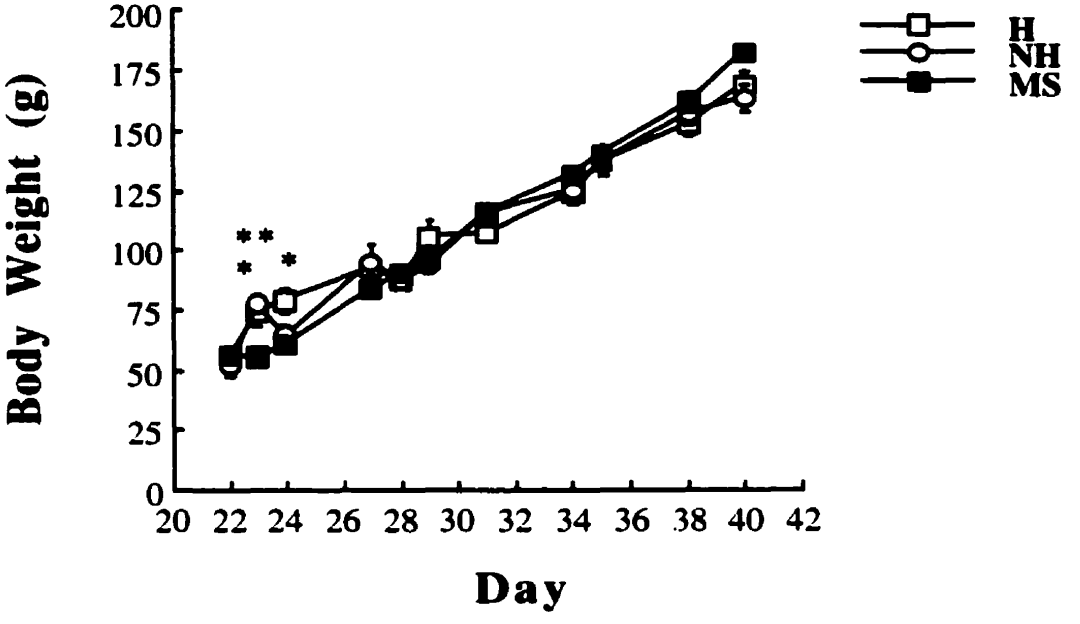

Figure 2. Mean (± SEM) body weights taken from postnatal day 22 (at weaning) until day 40 of life in males that were separated for 15 minutes (H) or 180 minutes (MS) per day from their mother from day 1-14 or left completely undisturbed (NH). N=20 per treatment
* H significantly different from MS; ** NH significantly different from MS.

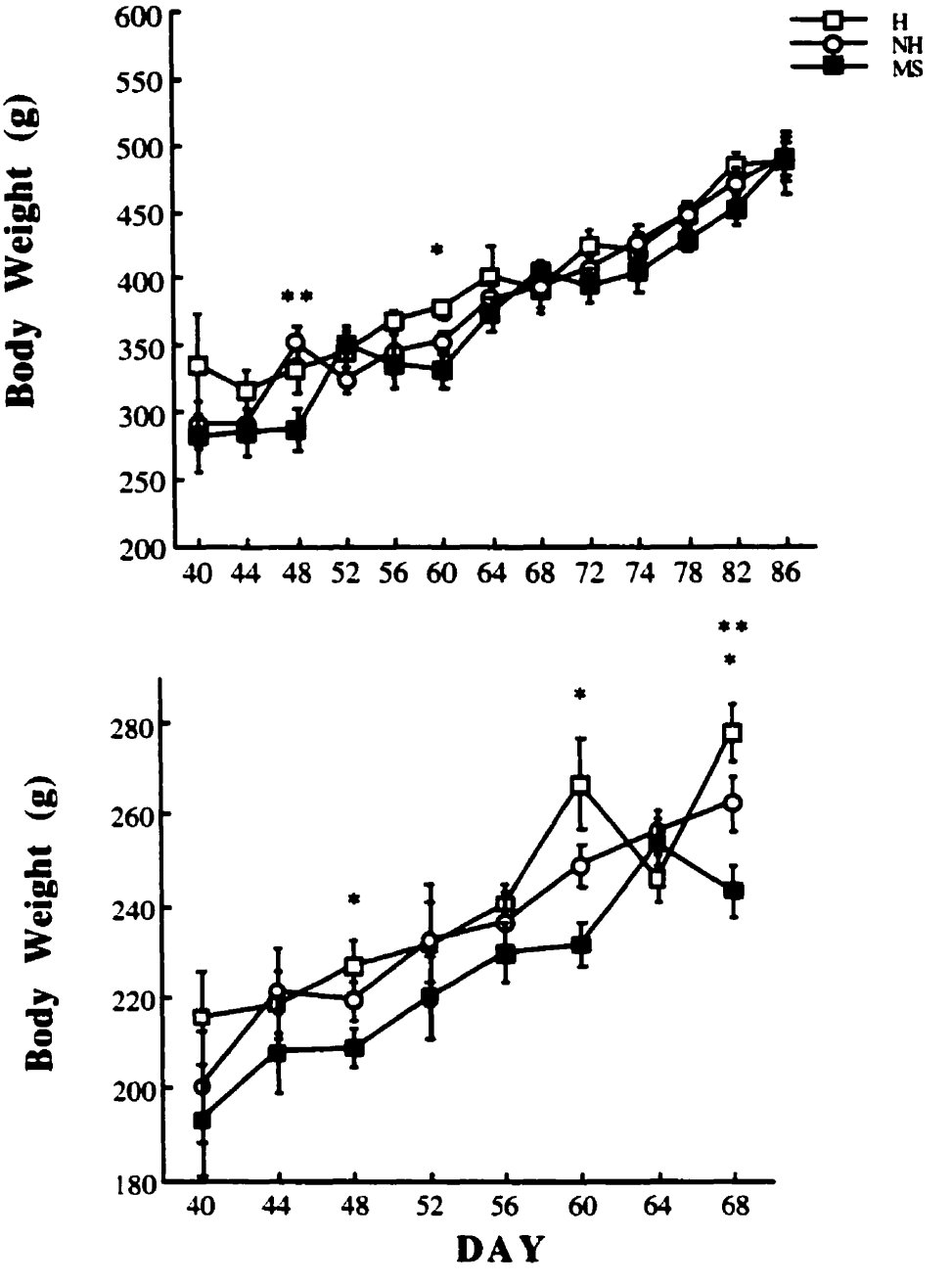

Figure 3. Mean (± SEM) body weights taken from postnatal day 40 until day 86 of life in males (top panel, n=10 for each treatment) and day 68 for females (bottom panel, n=10 for each treatment) that were separated for 15 minutes (H) or 180 minutes (MS) per day from their mother from day 1-14 or left completely undisturbed (NH). * H significantly different from MS; ** NH significantly different from MS.

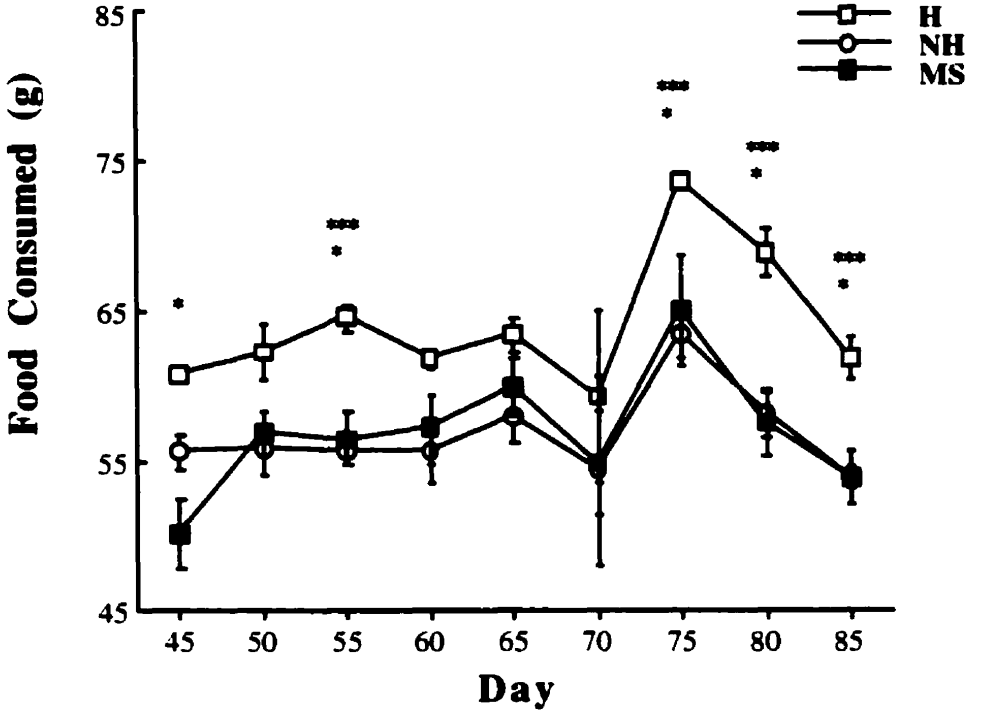

Figure 4. Mean (± SEM) daily food consumption in male H, NH and MS (n=16 per group) rats measured every second day from days 45-85 of life. * H significantly different from MS; *** H significantly different from NH.

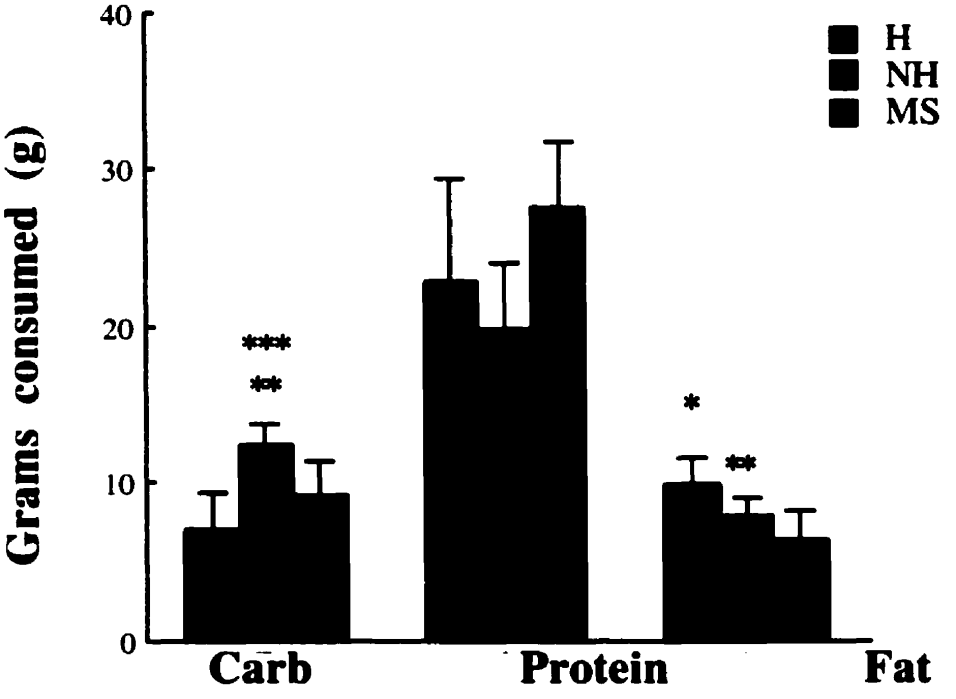

Figure 5. Mean (± SEM) mean daily carbohydrate (carb) (g), protein (g) and fat (g) consumed measured over one week (n=16 per group) in 3 month old rats. * H significantly different from MS; ** NH significantly different from MS; *** H significantly different from NH

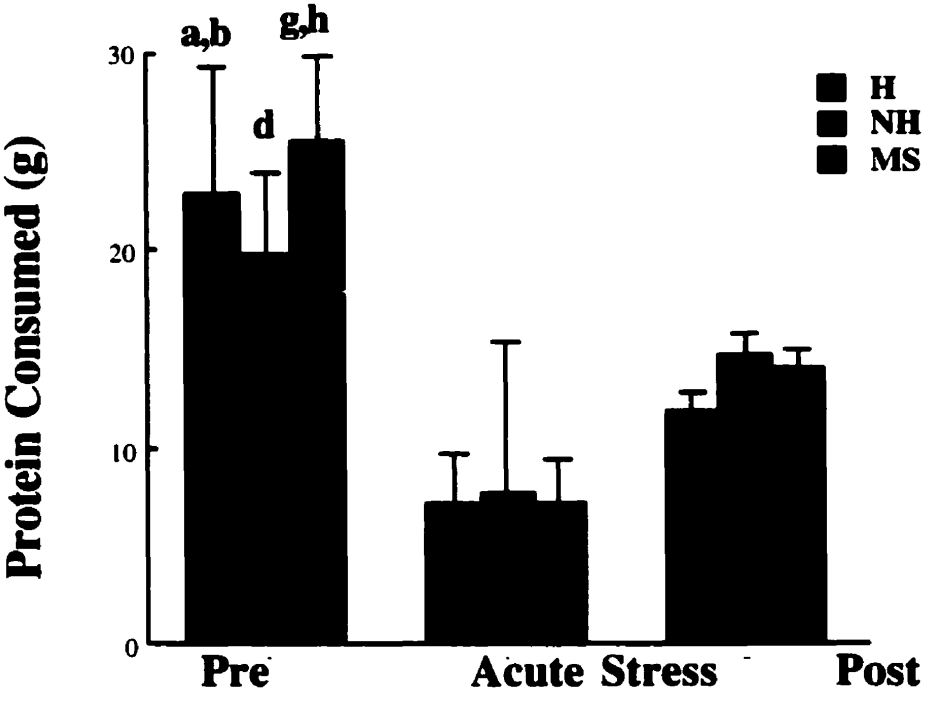

Figure 6. Mean (± SEM) protein (g) consumed 1 week before ("pre"), on the day of ("acute stress") and for 7 days following ("post") an acute stress in H, NH and MS rats (n=16 in each group). (a) H baseline different from H stress; (b) H baseline different from H post; (d)NH baseline different from NH stress; (g) MS baseline different from MS stress; (h) MS baseline different from MS post.

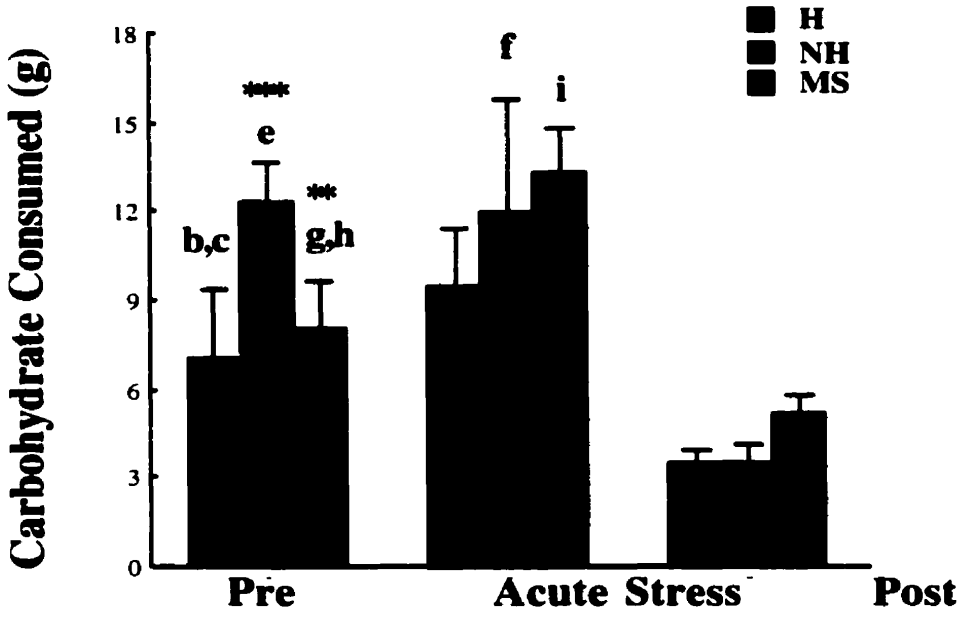

Figure 7. Mean (± SEM) carbohydrate (g) consumed 1 week before ("pre") , on the day of ("acute stress") and for 7 days following ("post") an acute stress in H, NH and MS rats (n=16 in each group). * *NH significantly different from MS; *** H significantly different from NH. (b) H pre sig different from H post; (c) H stress sig different from H post; (e) NH pre sig different from post; (f) NH stress sig different from NH post; (g) MS pre sig different from MS stress; (h) MS pre sig different from MS post; (i) MS stress sig different from MS post.

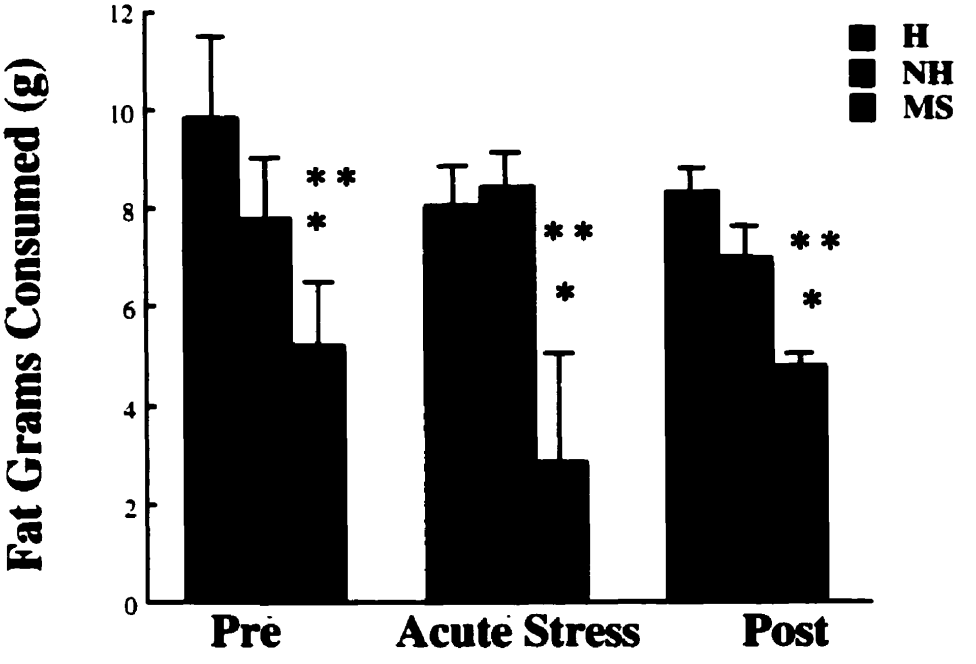

Figure 8. Mean (± SEM) fat (g) consumed 1 week before ("Pre") . on the day of ("acute stress") and for 7 days ("post") following an acute stress in H, NH and MS rats (n=16 in each group). * H significantly different from MS; ** NH significantly different from MS.

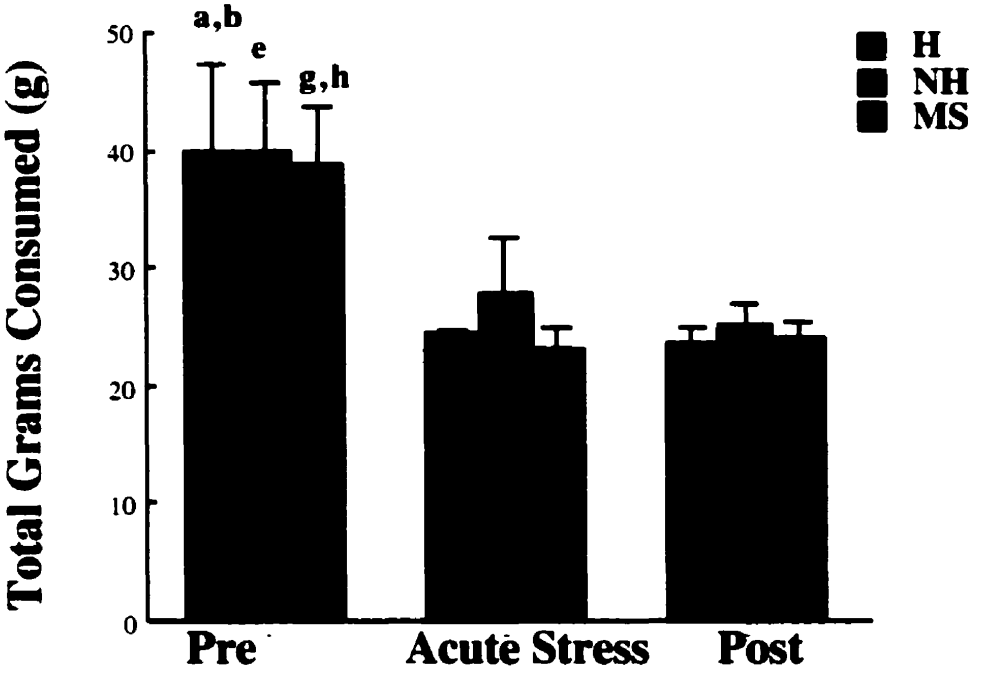

Figure 9. Mean (± SEM) total (protein, carbohydrate, fat combined) daily grams consumed for one week before ("pre"), during ("acute stress") and for one week following ("post") a period of acute stress; (a) H pre sig different from H stress; (b) H pre sig different from H post; (e) NH pre different from NH post; (g) MS pre sig different from MS stress; (h) MS pre significantly different from MS post. N=11 in each group.

Table 1. % of total grams consumed by H/NH/MS rats before, during and for one week following an acute stress

Pre

Group	Total Grams Consumed	% Protein	% Carbohydrate	% Fat
Handled	39.8 b	53.1 *	21.5	25.4 * b
Non-Handled	39.9	47.7 **	32.6	19.7
Maternally Sep	38.7	64.5	21.8	13.8 h

Acute Stress

Group	Total Grams Consumed	% Protein	% Carbohydrate	% Fat
Handled	24.6 a	28.9 a	38.4 a, c	32.7 *
Non-Handled	27.9	23.7 d	45.9	30.4 **, d
Maternally Sep	23.2 g	29.9 g,i	57.1 g,i	13.0

Post Stress

Group	Total Grams Consumed	% Protein	% Carbohydrate	% Fat
Handled	23.5	49.1 *, ***	15.4	35.5 *, ***
Non-Handled	25.1 e	58.0 e,f	13.8 e,f	28.1 **, e
Maternally Sep	23.9 h	58.7	21.0	20.4

All at $p<0.05$ or better:

* H vs MS

** NH vs MS
***H vs NH
a H pre vs H stress
b H pre vs H post
c H stress vs H post
d NH pre vs NH stress
e NH pre vs NH post
f NH stress vs NH post
g MS pre vs MS stress
h MS pre vs MS post
i MS stress vs MS post

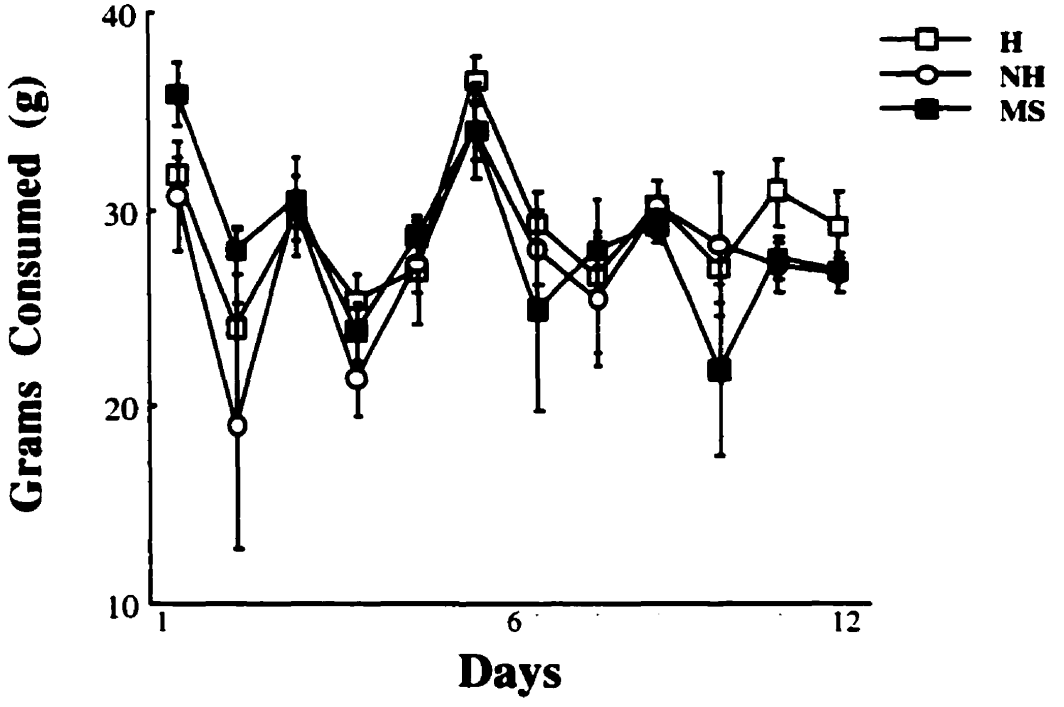

Figure 10. Mean (± SEM) daily food (chow) consumed over 12 days in 9-month old H, NH and MS male rats (n=20 per group).

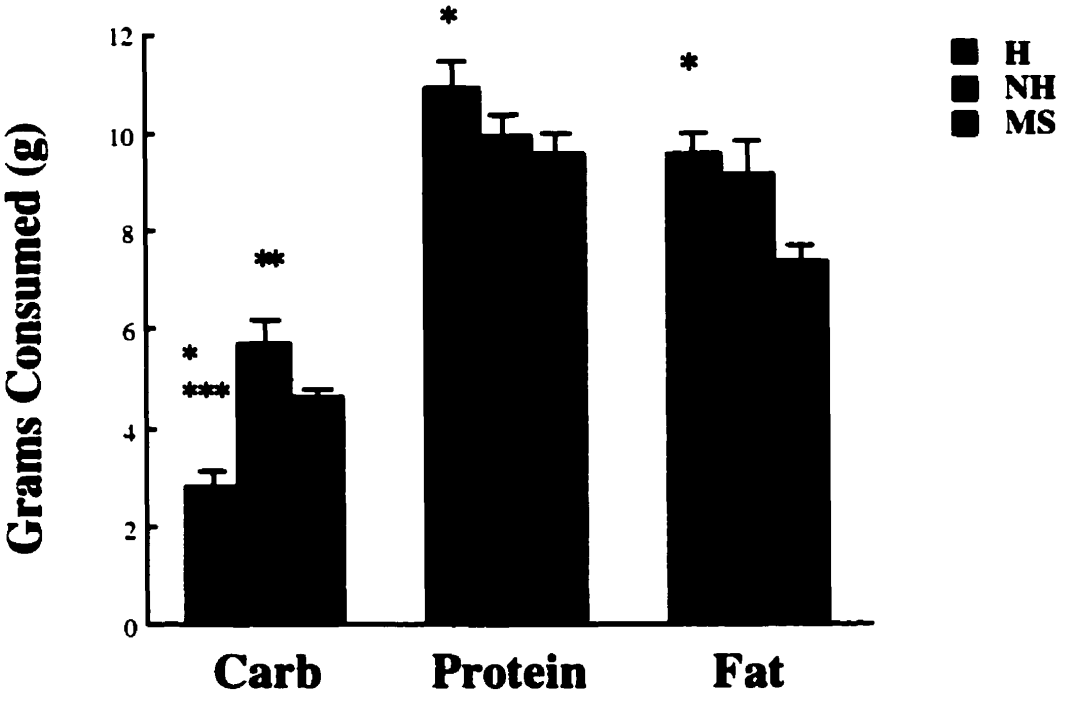

Figure 11. Mean (± SEM) mean daily carbohydrate (g), protein (g) and fat (g) consumed, measured over one week (n=11 per group) in 9 month old rats. * H significantly different from MS; ** NH significantly different from MS; *** H significantly different from NH.

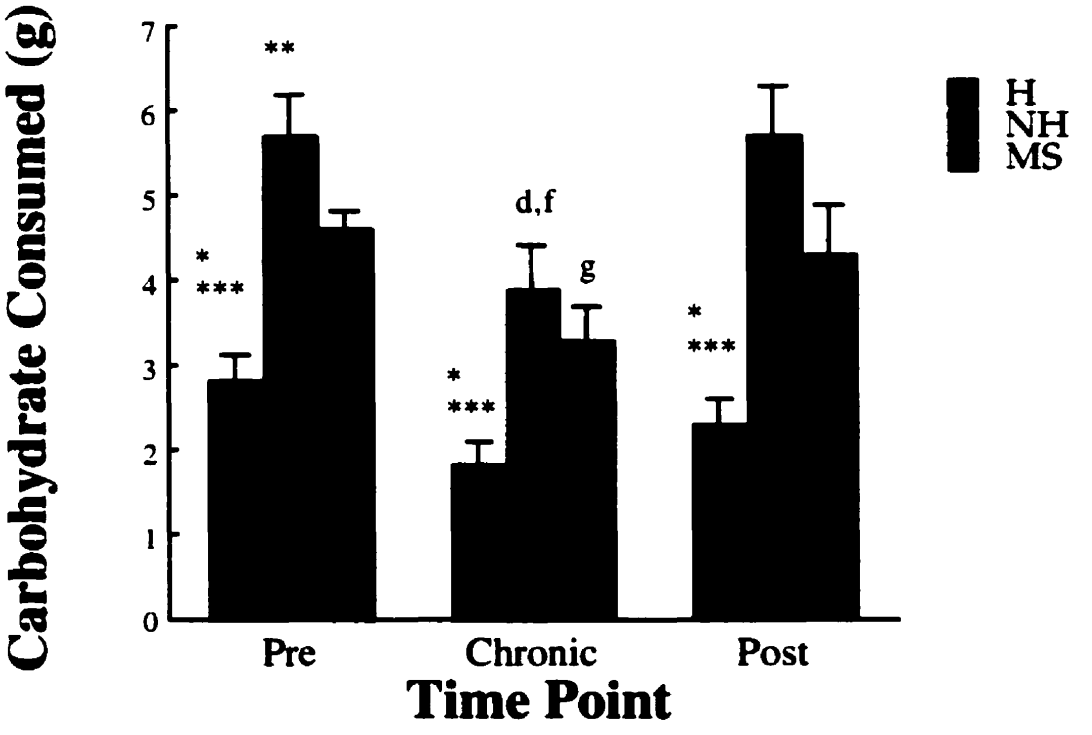

Figure 12. Mean (±SEM) daily carbohydrate (g) consumed for 1 week ("pre") before, during ("chronic") and for one week following ("post") a 21 day chronic stress. *H significantly different from MS; ** NH significantly different from MS; ***H significantly different from H.;(d) NH pre sig different from NH stress; (f) NH stress sig different from NH post; (g)MS pre sig different from MS stress. N=11 in each group

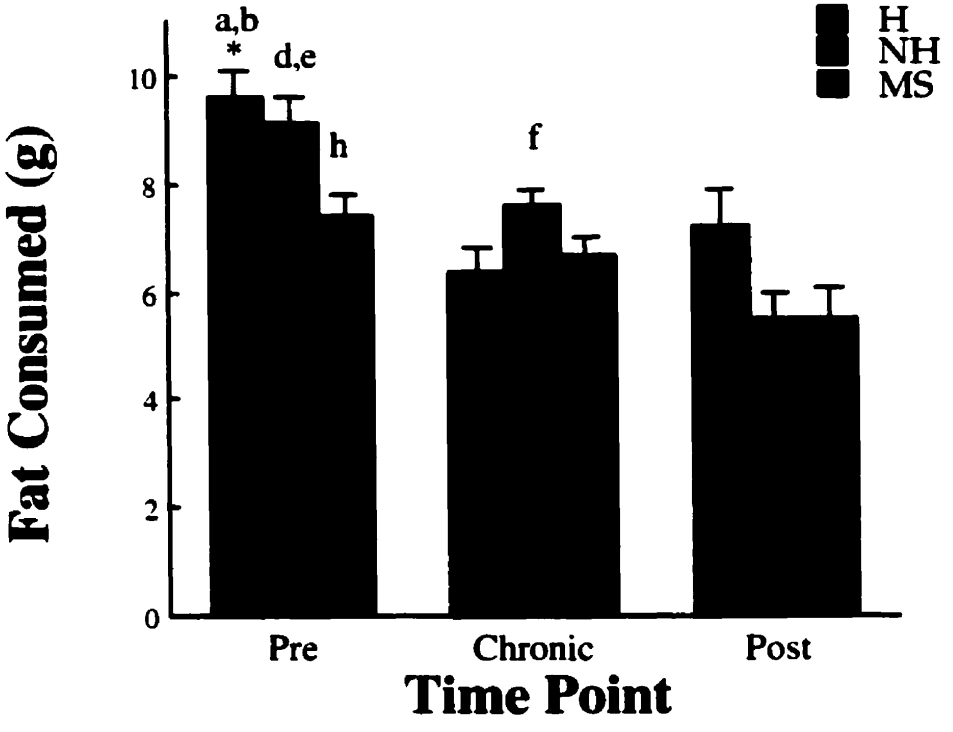

Figure 13. Mean (±SEM) daily fat (g) consumed for 1 week ("pre") before, during ("chronic") and for one week following ("post") a 21 day chronic stress. *H significantly different from MS; (a) H pre sig different from H stress; (b) H pre sig different from H post; (d) NH pre significantly different from NH stress; (e) NH pre sig different from NH post; (f) NH stress sig different from NH post; (h) MS pre significantly different from MS post. N=11 in each group.

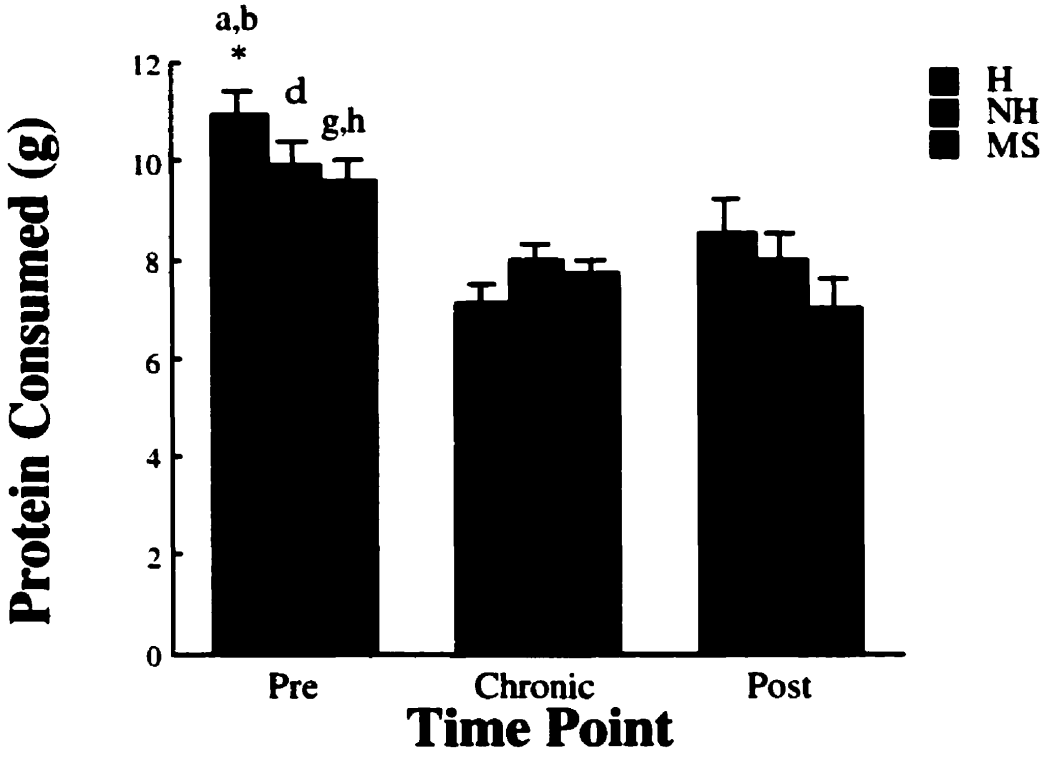

Figure 14. Mean (±SEM) daily protein (g) consumed for 1 week ("pre") before, during ("chronic") and for one week following ("post") a 21 day chronic stress. *H significantly different from MS; (a) H pre sig different from H stress; (b) H pre sig different from H post; (d) NH pre significantly different from NH stress; (g) MS pre sig different from MS stress; (h) MS pre significantly different from MS post. N=11 in each group.

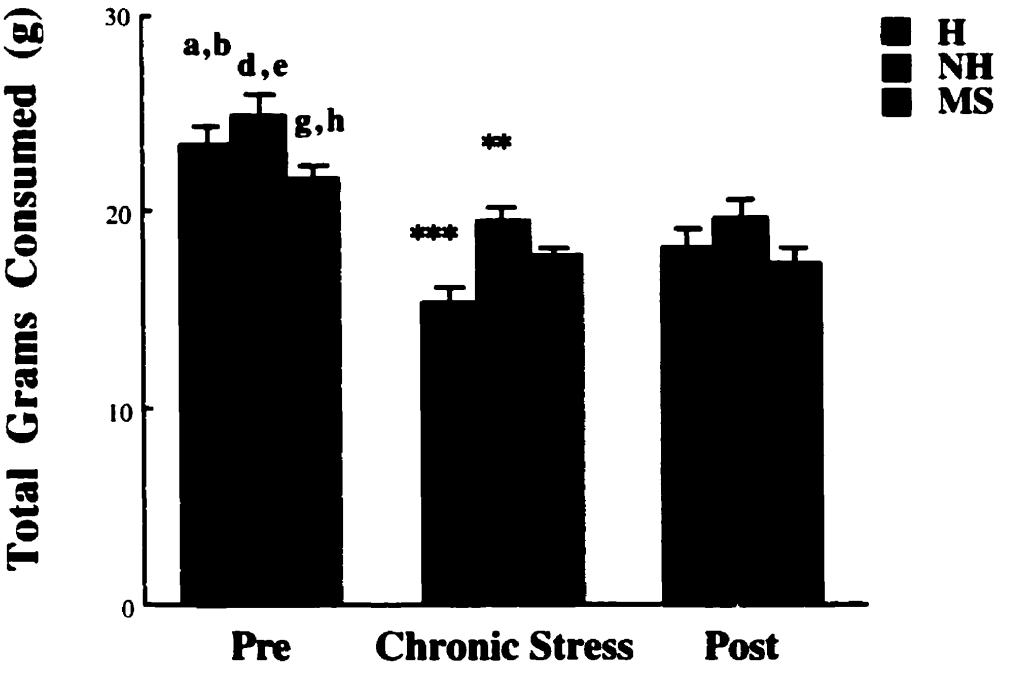

Figure 15. Mean (± SEM) total (protein, carbohydrate, fat combined) daily grams consumed for one week before ("pre"), during ("chronic stress") and for one week following ("post") a 21 day period of chronic stress. **NH significantly different from MS; *** H different from NH;(a) H pre sig different from H stress; (b) H pre sig different from H post; (d) NH pre significantly different from NH stress; (e) NH pre different from NH post; (g) MS pre sig different from MS stress; (h) MS pre significantly different from MS post. N=11 in each group.

Table 2. Percent (%) of total grams consumed by H/NH/MS rats before, during and for one week following a 3-week chronic stress

Pre

Group	Total Grams Consumed	% Protein	% Carbohydrate	% Fat
Handled	23.3 b	47.0 ***	10.9 ***	42.1 ***
Non-Handled	24.8 **	40.2 **	23.1	36.7
Maternally Sep	21.7 h	44.4	20.6 *, h	35.0 *

Chronic Stress

Group	Total Grams Consumed	% Protein	% Carbohydrate	% Fat
Handled	15.5 a	46.2 ***	11.7 ***	42.1
Non-Handled	19.5 ***, d	41.4	19.5	39
Maternally Sep	17.7 g	43.3	17.6 *, i	39 i

Post Stress

Group	Total Grams Consumed	% Protein	% Carbohydrate	% Fat
Handled	18.1	45.6	14.6 ***	39.8 ***
Non-Handled	19.6 e	42.5	28.6 f	29.0 f
Maternally Sep	17.3	42.0	26.3 *	31.7 *

All at $p<0.05$ or better:

* H vs MS

** NH vs MS
***H vs NH
a H pre vs H stress
b H pre vs H post
c H stress vs H post
d NH pre vs NH stress
e NH pre vs NH post
f NH stress vs NH post
g MS pre vs MS stress
h MS pre vs MS post
i MS stress vs MS post

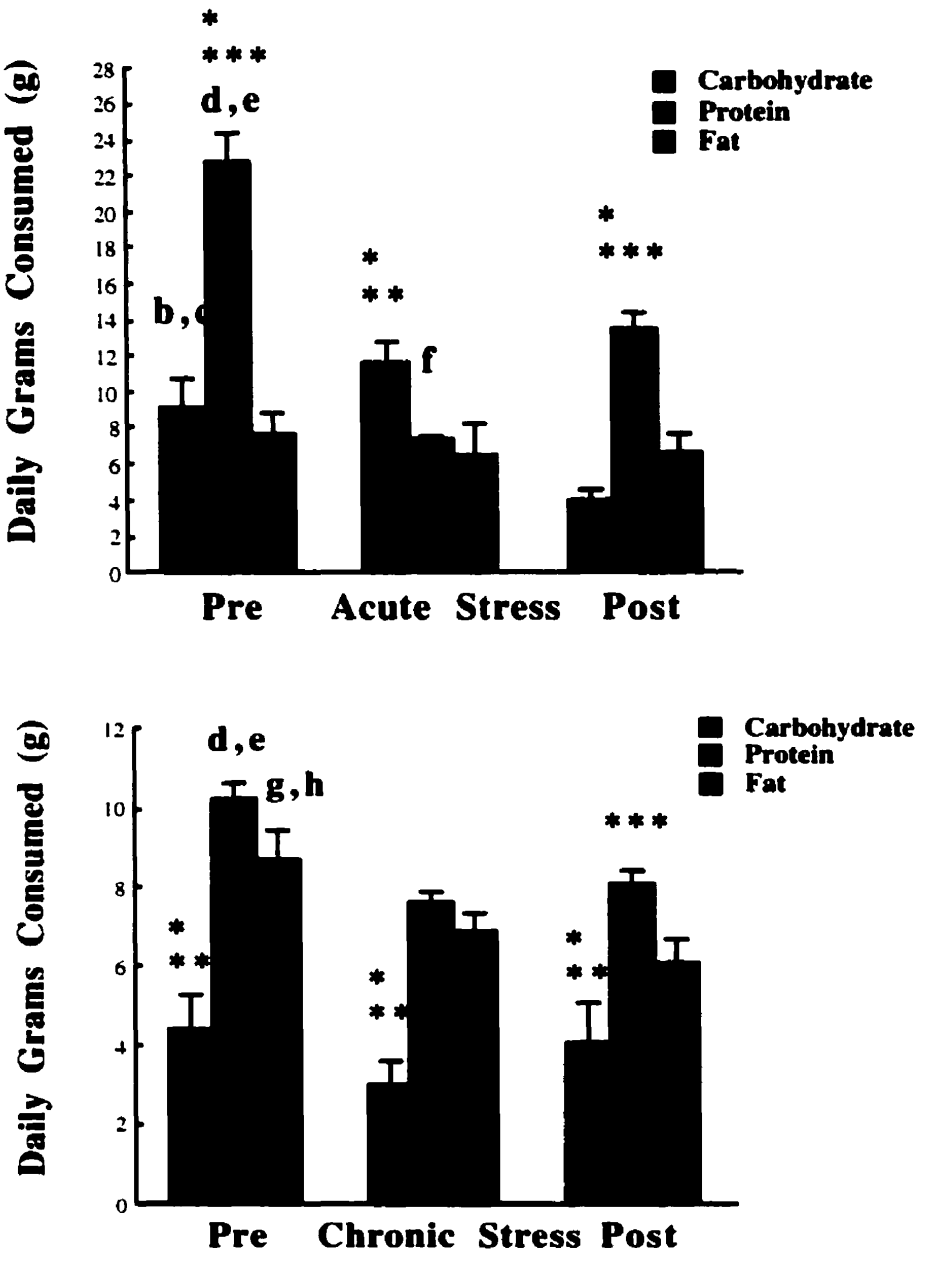

Figure 16. Mean (± SEM) daily macronutrients consumed before ("pre), during ("Acute Stress or Chronic Stress") and following ("Post") an acute (top panel) or 21-day chronic stress (bottom panel). * carbohydrate significantly different from protein; ** carbohydrate significantly different from fat; *** protein significantly different from fat; (b) pre carbohydrate vs stress carbohydrate; (c) stress carbohydrate vs post carbohydrate; (d) pre protein vs stress protein; (e) pre protein vs post protein; (f) stress protein vs post protein; (g) pre fat vs stress fat; (h) pre fat vs post fat.

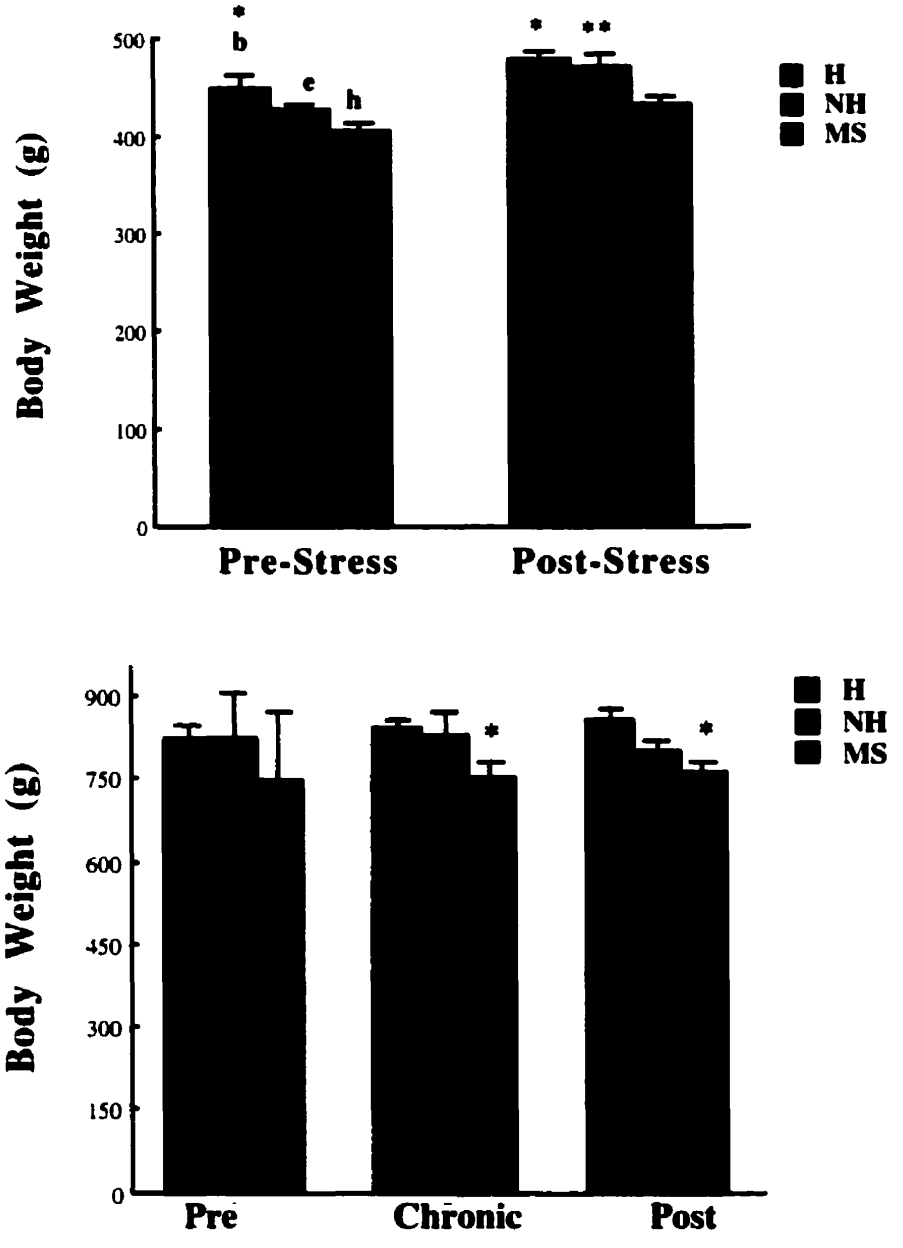

Figure 17. Mean (± SEM) body weights from H, NH and MS males (n=11 in each group) taken once per week 1 week before, during and for 1 week following a one hour acute stress (top) and 21-day variable chronic stressor regimen (bottom). * H significantly different from MS; ** NH different from MS; (b) H pre different from H post; (e) NH pre different from NH post; (h) MS pre different from MS post

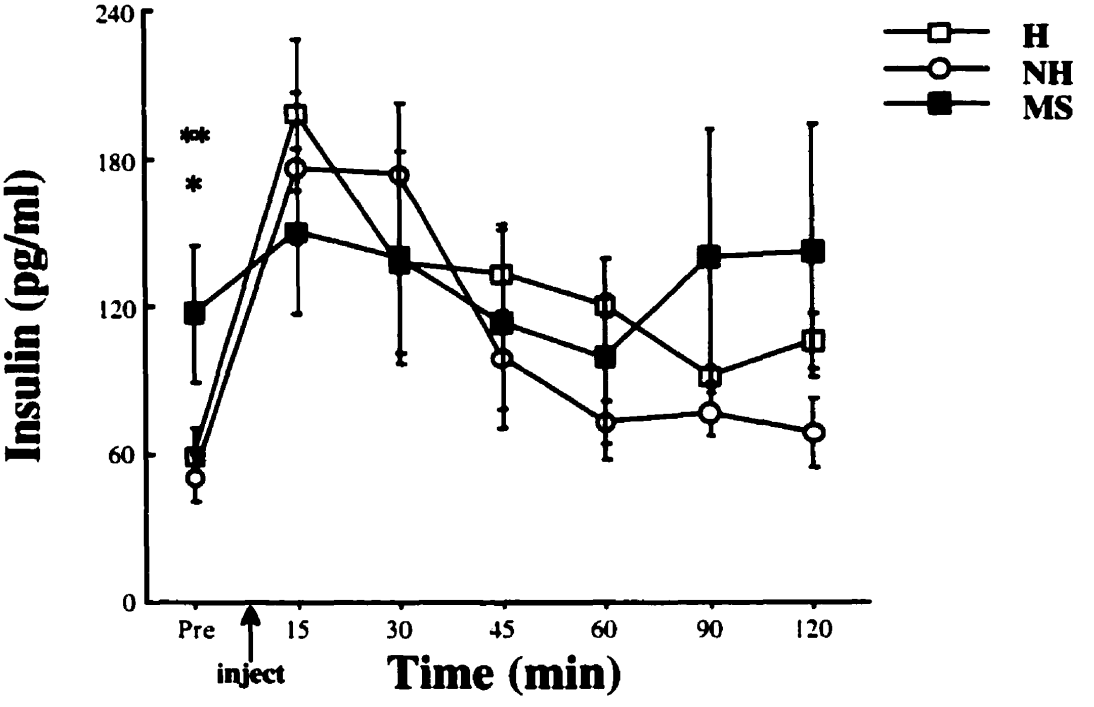

Figure 18. Mean (± SEM) plasma insulin (pg/ml) levels in 10-month old H, NH and MS rats (n=8 per group) after a 12 hour period of food deprivation ("pre") and 15, 30, 45, 60, 90 and 120 minutes following a 2.5 mg/kg i.p. glucose injection ("inject"). * H sig different from MS; **NH significantly different from MS.

Discussion

From postnatal days 1-85 H animals gain body weight faster and consume significantly more food than both NH and MS rats. Both NH and H rats show an endogenous preference for fat, when compared to MS rats, while NH rats also show a preference for carbohydrate when compared to MS and H rats. Acute stress diminishes intake of protein but leaves fat and carbohydrate consumption intact. Following acute stress animals do not resume pre-stress intake of total food. Chronic stress generally diminishes intake of all macronutrients. MS rats generally weigh less than both H and NH rats but significantly less than H rats. Finally, MS rats have significantly elevated basal levels of insulin and show no insulin response to glucose challenge when compared to both H and NH rats.

In general, H animals weigh significantly more than MS rats during postnatal days 1-21; H and NH rats have similar body weights from days 22 onward and H animals continue to weigh more than MS rats well into adulthood. McIntosh et al. (1999) and Vallee et al. (1996) found that neonatal handling resulted in greater weight gain from postnatal days 1 up until day 90, when compared to control and MS rats . Kuhn and colleagues (1990) found that prolonged periods of maternal separation caused a suppression in growth hormone (GH); in contrast brief periods of separation caused an increase in plasma GH levels. MS-induced suppression of GH and the resultant failure to gain body weight may be due to repeated short-term bouts of food deprivation (pups can suckle a number of times within a 3-hour period); food deprivation has been shown to decrease mean 6 hour GH levels in adult rats (Tannenbaum et al,, 1979). Food deprivation can also cause an increase in CRF levels (Bray et al., 1990). Interestingly, maternal separation causes both immediate and permanent increases in hypothalamic CRH mRNA and content (Plotsky and Meaney, 1993; Ladd et al., 1996). Exogenous CRF administration is associated with reduced feeding (Gosnell et al., 1983; Heinrichs and Koob, 1992) and has been implicated in both rat and human anorexia (Licinio et al., 1996)

. Therefore the potential reduction in suckling associated with maternal separation may cause acute periods of food deprivation, which result in increases in CRF and a reduction in body weight. It is also possible that it is the maternal separation itself that causes immediate elevations in CRF, which then set the cascade of, reduced feeding and body weight gain in motion. A recent report by Merali et al. (1998) suggests that bombesin (BN)-like peptides play a role in the response to both stressful and appetitive events. Administration of BN markedly suppresses food intake (Flynn, 1989) and both BN and CRH are markedly released in response to stress and food ingestion (Merali et al, 1998). Merali et al. (1998) postulate that food ingestion may be interpreted as "stressful" by certain amygdaloid circuits; thus one possibility is that MS rats have higher levels of both CRF and BN-like peptides and as a result, satiate faster, consume less food and have lower body weights.

As adults, MS rats show a prolonged B and ACTH response to acute stress (Plotsky and Meaney, 1993; Ladd et al., 1996). This brings up the potential for an interaction between the GCs and the GH/insulin-like growth factor (IGF) axis in these animals. Excessive GCs impair longitudinal growth and cause osteoporosis (Bloodgett et al. 1956). Long-term high-dose GCs alter GH pulsatility and decreased GH secretion from the hypophysis through an elevation in hypothalamic somatostatin tone in animals (Kovaks et al., 1991) and humans (Wehrenberg et al., 1990). Dexamethsaone treatment reduced GH-dependent IGF-1 mRNA in the liver (Luo and Murphy, 1989). A recent study by Jux et al. (1998) demonstrated that GCs decreased basal, GH- and IGF-1 stimulated chondrocyte cell replication, suggesting that GC-induced suppression of growth may be mediated, in part, by inhibition of IGF secretion. Thus, as MS rats most likely show both a greater lifelong exposure to circulating stress-induced increases in GCs, the finding that they weigh significantly less both early on in development and later in life is not surprising. Chronically elevated GCs may have potent effects on the somatotropic hormone axis, resulting in alterations in growth.

A number of other peptides may interact with CRF and GH to cause both hypophagia and reduced weight gain. For example NPY is known to be activated in response to periods of food deprivation and increased metabolic demand (Hanson et al., 1997). NPY stimulates CRH gene expression in the PVN (Suda et al., 1993) and NPY injected into the PVN stimulates ACTH secretion (Harfstrand et al., 1987). CRF inhibits NPY synthesis and release (White, 1993; Van Huijsduijnen et al., 1993) and suppresses the release of GH (Taylor and Fishman, 1988); GH and NPY both stimulate food intake (Krysiak et al., 1999). Interestingly insulin decreases the level of NPY in the arcuate nucleus; our MS rats showed higher levels of unstimulated insulin, which may have a direct effect on decreasing levels of NPY and reducing food intake and body weight gain. Insulin and GH also interact; for example the streptozotocin diabetic rat is GH-deficient (Zapf, 1998). In fact, treatment with moderate to low doses of GH improved the insulin sensitivity in men who had shown evidence of insulin resistance (Johannsson and Bengtsonn, 1999). Therefore, elevated CRF combined with increased GCs may be involved in the emergence of insulin insensitivity, blunted GH and NPY; ultimately this may result in lowered food consumption and body weight gain.

Transient hypothermia is induced during the separation of rat pups from their mothers. This may then cause elevations in levels of plasma T3 and T4 (Sharma and Meaney, unpublished results) which enhance fat metabolism and can result in weight loss. However, prevention of hypothermia does not eliminate the effects of handling on HPA function (Sullivan et al., 1988). Instead, when pups are licked by their mother small increases in body temperature occur which could induce thyroid hormone release (Levine, 1994). In fact, Liu et al. (1997) showed that mothers of H pups showed increased licking and grooming of pups and arched back nursing postures which later correlated with reduced plasma ACTH and B responses to stress and reduced hypothalamic CRH mRNA. Therefore it seems unlikely that the lower body weight seen in MS pups are a result of increased thyroid hormones since increased thyroid hormones emerge as a result of

increased tactile stimulation from the mother and some recent evidence suggests that MS pups receive substantially less licking and grooming than both H and NH rat pups (Meaney et al., unpublished observations). Males weighed more than females from days 40-68 but we observed the same trend in females as in males, namely that H rats weighed significantly more than both NH and MS rats at numerous points.

We found that manipulating the neonatal environment has distinct effects on baseline food choice in rats tested at both three and nine months of age. H and NH rats consumed more fat than MS rats, and NH rats consumed more carbohydrate than both H and MS rats. A number of studies have suggested that when allowed to self-select from pure macronutrient diets (protein, carbohydrate and fat), rats from the same genetic strain can be categorized into three subpopulations: high-fat consumers, high-carbohydrate consumers or high protein consumers (Shor-Posner et al., 1991). So it is not entirely surprising to find that our rat population exhibited distinct preferences as well. Tempel and Leibowitz (1994) have proposed a number of neural signals that may be involved in macronutrient choice. Fat consumption and metabolism are regulated by the hypothalamic neuropeptide galanin (GAL); when injected into the hypothalamus of satiated rats, GAL stimulates food intake and in some conditions, preference for dietary fat (Tempel et al., 1988; Crawley et al., 1990). Moreover in normal weight rats given a choice of macronutrient diets, GAL levels and gene expression are positively correlated with the ingestion of fat but not of carbohydrate and protein (Akabayashi et al., 1994). Central administration of GAL has been shown to reduce circulating levels of B and insulin (Tempel and Leibowitz, 1990) and stimulate the release of GH (Murakami et al., 1987). More recent work suggests that increased GAL levels induced by ingestion of a high-fat diet are accompanied by lower levels of both B and insulin (Leibowitz et al., 1998). Previous work has shown that H rats exhibit an attenuated increase in B in response to stress and a faster return to basal plasma B concentrations following the termination of the stressor (Meaney et al., 1995); this may be due, in part to differences in circulating GAL.

H rats in this study also have significantly lower circulating insulin, which again might be a reflection of circulating GAL. Injections of B cause a preferential rise in the ingestion of carbohydrate (Tempel and Leibowitz, 1993); NH rats show reduced negative feedback efficacy in that their return of plasma B levels back to baseline following acute stress is slower (Meaney et al., 1995). While basal B levels in H and NH rats are similar, cumulative exposure to GCs over the lifespan seems to be greater in NH versus H rats (Meaney et al., 1988). Higher carbohydrate consumption in NH rats may emerge, in part, due to a higher overall level of circulating GCs.

Both acute and chronic stressors have been shown to attenuate food intake; therefore it is not surprising that both the acute and chronic stressor suppress food consumption in general. In addition, animals tend to continue to suppress their food intake for the week following acute and chronic stress, although they do not diminish their consumption further. When we examined the particular macronutrients, protein consumption was reduced during stress in all rat groups. MS and H rats continued to significantly suppress their protein intake during the week following stress. NH rats consumed less protein during the post-stress compared to the pre-stress period, suggesting that acute stressors have prolonged effects on protein consumption. Carbohydrate consumption either remained the same (in NH rats) or increased (in H and MS rats) in response to acute stress. Following stress, carbohydrate consumption is dramatically reduced in all groups of rats. It is not entirely surprising that during stress, rats will consume more carbohydrate to replenish lost energy reserves and consume the macronutrient that is most rapidly converted into utilizable energy. Carbohydrate is the macronutrient that is most rapidly converted to glucose (Kumar and Leibowitz, 1988) and the response to stress demands immediate glucose reserves. In fact a number of studies have suggested that exogenous administration of B causes a preferential intake of carbohydrate (Tempel and Leibowitz, 1994) and carbohydrate is most heavily consumed at the onset of the dark period, when B levels are peaking (Kumar and Leibowitz, 1988).

What is also striking is that H rats, who show the lowest stress-induced peak in B, also consume the least amount of carbohydrate in response to stress. Once the stress is terminated, all rats will suppress their intake of carbohydrate having restored their energy supply. Stress does not seem to have any effect on fat consumption, although H and NH rats consume more fat than MS rats during all three periods.

Before the acute stress, H rats weigh significantly more than MS rats. Following the acute stress all rats continue to gain weight but at this point both H and NH rats weigh more than the MS rats. Acute stress does not seem to have an effect on body weight gain, which has been shown previously. However the early environmental manipulations affect body weight which may reflect differences in CRF. However since H rats prefer fat, and consume more of it and since the fat diet has the greatest amount of calories, the increased body weight seen in H rats may reflect their greater consumption of fat.

When rats are tested at 9 months of age, there are no differences in regular chow consumption, despite differences in body weight (NH rats weigh significantly more than MS rats). Interestingly, similar patterns of baseline macronutrient consumption persists in the rats when they are tested at 9 months of age. NH rats still consume significantly more carbohydrate than H and MS rats, H rats consume more protein and fat than MS rats and H rats consume significantly less carbohydrate than MS rats (H rats consumed less carbohydrate at 3 months of age but it was not significant).

In response to chronic stress, all rats significantly reduced total food consumption and consumption remained suppressed during the post-stress period. The effect of repeated exposure to stress on food intake has produced discrepant results. Repeated immobilization of rats for 7 days inhibited daily food intake by 25-30% on all days of immobilization (Michajlovskij et al., 1988). Marti et al.(1994), exposed rats to handling, one hour of restraint or 1 hour of immobilization each day for 27 days; after 7 and 27 days, food intake and body weight were reduced in restrained and immobilized rats compared with undisturbed controls. The inhibition was greater on day 1 than on day 7 and 27

suggesting that there was some recovery of intake. Krahn et al. (1990) found similar results with 5 days of restraint.

To date, very few studies have examined the effects of stress on macronutrient selection. In one study, young rats subjected to 21-days of tail pinch stress did not change any aspect of intake (Bernardis and Bellinger, 1981). Zhou et al. (1999) have recently shown that 5 days of repeated restraint stress caused a significant decrease in carbohydrate, a significant increase in protein and no change in fat consumption. We also found a significant decrease in carbohydrate consumption during chronic stress, but only in NH and MS rats. Once the stressor period was terminated, both NH and MS returned their carbohydrate consumption back to pre-stress levels. H rats consumed the same amount of carbohydrate throughout all time periods but consumption was significantly lower than NH and MS rats at each time point. This raises a number of issues. First, while acute stress may necessitate immediate reserves of energy to cope with the stressor, adaptation over 21 days of chronic stress may occur. Thus rats may not require immediate glucose (in the form of carbohydrates) during chronic stress, but once the stress is over, may return carbohydrate consumption back to baseline levels. Again, this may be related to circulating B levels; H rats mount an overall lower B and ACTH response to acute stress and with 21 days of intermittent cold show a reduced B response the 21st bout of cold and an overall reduced response to a novel, heterotypic stress when compared to NH rats (Bhatnagar and Meaney, 1995), suggesting a greater adaptation to chronic stress and concomitant minimal activation of the HPA axis.. Thus, lower overall B secretion during chronic stress may be related to lower carbohydrate consumption.

Fat consumption is decreased by all rats in response to the chronic stress period, with the exception of the MS rats. MS rats ultimately reduce their fat consumption following chronic stress relative to what they consumed before the chronic stress period. NH rats continue to suppress fat consumption even further following stress. A number of studies by Wilner's group (Wilner, 1997; Wilner et al., 1998) have demonstrated that

during chronic mild stress, the rewarding properties of food are decreased and these effects can persist for up to 3 months. Rewarding processes have been thought to play a role in sustaining the activity of the behavioral systems which maintain feeding behavior once it has begun and may be associated with the pleasure derived from eating, an area where opioids might have some influence. Opioids generally stimulate the consumption of the preferred macronutrient as opposed to stimulating any one macronutrient per se. In a number of studies, rats were classified as high-fat, high-carbohydrate or intermediate preferrers based on daily intake patterns. An injection of morphine stimulated the intake of the preferred macronutrient in fat or carbohydrate preferring groups, while nonsignificantly increasing fat and carbohydrate in the intermediate groups (Gosnell et al., 1990). H rats consumed the most fat at baseline and showed the biggest decrease in fat consumption during stress, while MS and NH showed the most substantial decrease of their preferred macronutrient, carbohydrate, in response to stress.

In the chronic stress experiment, H rats weigh significantly more than MS and H rats are the only group of rats that show some weight gain both during and following the chronic stress (NH rats lose weight during chronic stress while MS rats, don't lose weight but weigh less than H rats throughout). Bhatnagar and Meaney (1995) demonstrated that H rats show a greater adaptation to chronic stress than NH rats. This suggests that the NH rats in our study may have not adapted to the chronic stress and as a result, had higher circulating B levels. If B levels were in the catabolic range (Dallman et al., 1993) , then it is possible that the effects of B in both the NH and MS rats were to cause a reduction in body weight. Of course the other potential mechanism for the reduced body weight may be increased CRF levels in both MS and NH rats.

The question that remains is what are some of the implications for these individual patterns of food consumption? While all animals show similar insulin profiles in response to a glucose tolerance test, MS rats have significant higher basal insulin levels and show virtually no response to glucose injection. This is indicative of insulin insensitivity (Felig

and Berman., 1995). While it is difficult to interpret this elevation in plasma insulin as a consequence of particular dietary habits it is intriguing to speculate on what some of the sources for this elevation might be. Obviously higher circulating GC levels in MS rats might be partly responsible for increased insulin. This stems from data suggesting that a prolonged elevation in GCs can antagonize the effects of insulin. The excessive production of GCs can produce an insulin insensitivity (Brindley and Rolland, 1989) and increase insulin levels. Insulin levels are increased to try to compensate for the insulin insensitivity and the increased insulin can be atherogenic (Sleder et al., 1980; Tobey et al., 1982) and may increase the risk for the development of type 2 diabetes. This antagonism may also lead to a decreased uptake of LDL, one of the hallmarks of CAD. What is also of interest is that despite this difference in basal insulin, MS rats weigh significantly less than NH and H rats (before and after chronic stress, respectively). This suggests that it may not necessarily be the quality or quantity of what MS rats are consuming but rather the hormonal milieu (high insulin and B) that could contribute to the development of insulin insensitivity and ultimately diabetes.

In sum, these data suggest that maternal separation causes both short and long-term decreased body weight gain and food intake while handling causes increased body weight gain and food intake. Neonatal manipulations have distinct effects on endogenous food preferences, with H rats preferring fat and NH rats preferring carbohydrate. While both acute and chronic stress generally decrease food consumption, acute stress causes an increase in the consumption of carbohydrates, perhaps as a substrate for immediate energy. Interestingly H rats do not increase their consumption of carbohydrate, perhaps due to their ability to cope more efficiently with the stressor. MS rats show evidence for reduced insulin sensitivity, which may be related to chronically elevated B levels; this insulin sensitivity is apparent in the absence of increased fat and carbohydrate intake and independent of body weight. Taken together these results suggest that the early environment can have profound and permanent effects on food intake and body weight

which may or may not contribute to differences in carbohydrate metabolism. This is in agreement with much of the clinical literature which has found a link between the rearing environment and the emergence of eating-related pathology.

The consequences of alterations in food preferences in response to stress cannot be underscored. Not only do these choices impact on the physiological response to stress (by their alterations in GC production) but particular foods themselves can modulate the psychological and emotional responses to the stressors themselves. We explored this possibility by examining alterations in dietary patterns and affective status in humans, in response to a chronic academic stress.

Study 2 The Effects of Chronic Academic Stress on Food Consumption and Affective Status in Humans.

Several lines of evidence suggest that stress contributes to the development of coronary artery disease (CAD), NIDDM and stroke. The mechanisms through which stress leads to these diseases are, in part, mediated by the effects of stress hormones, GCs and catecholamines. Acute elevations in blood pressure and heart rate during stress may promote damage to arteries by increasing sheer stress (Bord, 1983). Emotional stress leads to increased catecholamines, which may result in the activation of platelets, providing a background for the development of atherosclerotic lesions (McCann et al., 1990). Chronic and/or repeated stressors have also been shown to raise plasma lipid and cholesterol levels in rodent non-human primate and human populations (Dimsdale and Herd, 1982). Plasma cholesterol levels measured prior to a final examination in medical students were 20% higher than cholesterol levels measured 48 hours after the exam (Dreyfuss and Czaczkes, 1959). Approaching deadlines for tax accountants raised serum cholesterol levels (Friedman et al., 1958); chronic unemployment had similar effects (Kasl al., 1968). Trevisan et al. (1986) found that participants screened for risk factors for CAD had a higher heart rate, elevated serum cholesterol and elevated triglyceride levels following a major

earthquake than matched participants screened before the catastrophic event, again suggesting that the perception and experience of stressful events can have potent effects on serum lipids. In rats, stressor exposures, including uncontrollable shock (Berger et al., 1980; Servatius et al., 1994), and even acute immobilization (Ruiz de Gordoa et al., 1994) result in elevations in plasma cholesterol and/or triglycerides. In baboons, social subordinance, a chronic social stressor, resulted in reduced HDL-C and apolipoprotein A-1 concentrations, factors known to be antiatherogenic (Sapolsky and Mott, 1987). Therefore these studies suggest that stress could be one pathogenic factor contributing to hyperlipidemia and atherosclerosis and thus a greater risk for disease.

The question that necessarily follows is what are some of the contributing factors that lead to these stress-induced elevations in plasma lipids? ACTH and cortisol are potent stimulators of lipolysis (Fain et al., 1979), the breakdown of triglycerides in adipose tissue and the eventual liberation of free fatty acids (FFA) into the plasma (Felig et al., 1995) . Exogenous administration of GCs in the stress range caused a 60% increase in plasma palmitate (a long chain FFA) concentrations (Fain et al., 1979); this study was done with a pancreatic clamp technique in order to prevent changes in insulin, glucose and growth hormone which may have obscured the effect of F on lipolysis. Short-term administration of GCs and epinephrine (EPI) to rats causes hypercholesterolemia (Kunihara and Oshima, 1983; Brindley et al., 1993). Dexamethasone (DEX) a synthetic GC, increases the hepatic secretion of triglycerides, cholesterol and decreases levels of LDL receptors, which are primarily responsible for the clearance of LDL from circulation (Martin-Sanz et al., 1990). Interestingly, exogenous infusion of FFA activates the HPA axis by elevating both plasma ACTH and B in rats (Widmaier et al., 1992), suggesting a reciprocal relationship between FFA and B. Thus products of the HPA axis themselves, namely ACTH, B and EPI, may be at least partly responsible for the increases in plasma lipids seen following acute and chronic stressors.

Another possible candidate is the diet. The relationship between both acute and chronic stressors and dietary patterns has been characterized in human, non-human primate and rodent populations. Studies in humans have been contradictory, with some showing that food intake is unaffected by stress, and others demonstrating substantial effects of different stressors on patterns of food consumption. One of the first studies to examine this relationship found that while periods of high stress for tax accountants were associated with a rise in serum cholesterol, this was not attributable to changes in dietary habits during that time (Freidman et al., 1958). In another study, the food intake of 12 men awaiting hernia surgery was compared to their intake one month later (Bellisle et al., 1990). While anxiety levels were higher on the day before surgery than following recovery, food intake was unaffected by such stress. McCann and colleagues (1990) followed the dietary intake of individuals during periods of high and low workload. Total energy and fat intake were higher during the high-workload periods than during the quiescent periods. Niaura's group (1991) found that while accountants did not change their eating habits during the stressful tax season, they did consume more saturated fats following the tax deadline. A number of studies by Lindquist et al. (1995;1997) showed that under both home and job stress conditions, subjects were more likely to use "unhealthy eating" as a coping strategy. This raises an interesting issue regarding the use of food as a coping mechanism during stress. There is some evidence suggesting that dietary factors have can have a substantial impact on mood. For example, low levels of serotonin have been found in depressed patients; these patients also suffer from folate deficiency, which may influence the level of serotonin in the brain (Young, 1993). Carbohydrate and protein meals raise brain serotonin and tryptophan (Wurtman and Wurtman., 1995) which may serve to improve mood. Therefore it is feasible that food choice may be specific during times of stress as a means of coping with the situation; conversely it is possible that adrenal GCs secreted during stress may themselves direct the pattern of food selection, as high levels of GCs have been associated

with increased carbohydrate, fat and total calorie consumption (Dallman et al., 1993; Tempel, and Leibowitz 1994).

In rodents, acute stressors that have been used to assess the effects of stress on diet include tail pinch, restraint, immobilization, and acute treatment with CRF. Krahn et al. (1986) examined the effect of one hour of restraint on feeding behavior in food deprived rats. Restraint significantly reduced food intake relative to unrestrained controls. The effect of restraint stress on feeding has been replicated by a number of groups (Kennet et al., 1985a;b; Donohoe et al., 1987); in fact, Shibasaki et al. (1988) found that 90 minutes of restraint stress reduced food intake by rats to approximately 60% of control rats levels. In addition, restraint stress-induced reductions in feeding were partially dependent on the duration of restraint: 30 minutes of restraint was sufficient to reduce food intake and 60 and 90 minutes of restraint caused an even greater reduction in food intake relative to rats restrained for 30 minutes. Heinrichs and Koob (1992) found that restraint stress significantly reduced the intake of unfamiliar food in animals that had been protein deficient. Zylan and Brown (1996) found that 20-minute immobilization stress significantly reduced food intake in both male and female rats, although females did not consume more food with repeated stressor exposure (chronic and repeated stress effects on food intake will be discussed below). Ely et al. (1997) found that acute restraint did not produce any alterations in consumption of a sweet snack. Conversely, tail-pinch stress has been shown to cause hyperphagia (Robbins et al. 1977; Wallach et al., 1977; Morley et al., 1983; Kalra and Kalra, 1990). Therefore, these results suggest that the type of stressor, the type of food presented and the condition of the subject may have an impact on the feeding response to acute stress.

Chronic exposure to a variety of stressors of a certain severity has been shown to decrease food intake and body weight gain in the rat. Marti et al. (1994) subjected adult male rats to various chronic stressors of differing intensities and for different periods of

daily exposure. While a mild stimulus, such as one minute of daily handling for 27 days, had no effect on food intake and body weight, chronic restraint slightly reduced food intake and body weight gain and chronic, daily immobilization produced the greatest decrease in food intake and body weight gain (Marti et al., 1994). The responses were similar during the entire exposure period because the changes in body weight gain and food intake were of the same extent 1 or 4 weeks after the beginning of treatment. Thus habituation to these chronic stressors was not apparent in this study. In a second experiment, rats were exposed to 2-hour immobilization, every day for 14 days (Marti et al., 1994). Food intake was most dramatically decreased following a single 2-hour session (acute effects), but remained decreased on subsequent days of exposure to isolation stress. No major changes in the circadian rhythm of food intake was found in response to chronic immobilization, but the percent of food eaten by stressed rats between 0400-0800h was always lower than that of control rats. The authors also assessed the influence of the daily duration of exposure to the same stressor: while adrenal weights were greater in rats subjected to 240 minutes of immobilization stress when compared to rats exposed to 15 and 60 minutes of immobilization, all rats consumed similar amounts of food and showed similar reductions in body weight (Marti et al., 1994). In the study by Ely et al. (1997) animals that were restrained for one hour per day for 5 days per week for 50 days showed an increased ingestion of sweet food when compared to unstressed controls. Heinrichs et al. (1992) found that repeated tail pinch (once per day for 4 days) resulted in increased food consumption with each successive day and a decreased latency to eat between the first testing day and the fourth. Repeated cold stress resulted in increased food intake with decreased body weight gain (Kawanishi et al., 1997). In addition, the diurnal variation in body weight decreased. Kant and Bauman (1993) found that animals exposed to chronic, intermittent footshock decreased their food intake of both low and high sucrose pellets, although there was a greater decrease in pressing for the low sucrose pellet than the high sucrose pellet. Zylan and Brown (1996) found that with repeated immobilization stress,

both males and females continually increased their food intake with each successive session and females, in particular, showed an increase in food intake from the third to the fourth sessions, while males stabilized their intake between the third and last session. Taken together these studies suggest that the effects of stress on dietary patterns depends on the type, duration and intensity of the stressor as well as the gender and metabolic state of the subject.

Since it is well known that unhealthy diets contribute to CAD and are associated with the risk factors associated with heart disease, including elevations in plasma cholesterol, triglycerides, and decreased HDL and apolipoprotein A-1, it seems critical to evaluate dietary changes during periods of stress in evaluating the effects of stress on health and ultimately on the progression of disease. Previous work in this area is limited and studies that have been done are lacking. First, none of these studies measured both F levels and dietary patterns at the same time. In fact, the majority of the studies assessed subjective feelings of stress via questionnaires and not by directly measuring circulating levels of F. Kirschbaum et al. (1992) reported no correlation between F levels and personality traits suggesting that subjective reports from subjects to imply F levels are not always accurate. Second, dietary analyses were done for a short period of time only; this kind of analysis is limited since dietary patterns fluctuate across days and seasons. In general , dietary analyses have been completed by having subjects recall what they had consumed, leaving the possibility open for inaccurate, limited and fabricated details. Finally, many of these studies, especially those done in human subjects employed unrealistic, subjective, rare (in the case of an earthquake) and uncontrolled stressors. The stress should be fairly equal among subjects and should be a stress that commonly occurs. Furthermore, subjects need to be assessed during non-stressful periods in addition to the periods prior to, during and immediately following the termination of the stress. This allows the examination of some of the contributing factors to the response to the stress itself as well as an assessment of any long-term effects of the stress once it is terminated.

Therefore the purpose of the present experiment was to assess both the perceived and actual responses to academic stress and how these responses were related to dietary factors, gender and affective status.

Methods

Subjects/Preliminary Meeting

Eleven undergraduate students volunteered for the study. Three males and eight females were between the ages of 19-25 and enrolled as full-time undergraduate students at McGill University. Informed consent was obtained from all subjects. All students were required to attend a preliminary meeting with the experimenter prior to participation in the study. At this meeting, subjects received a thorough overview of the procedure: how to complete their daily dietary and activity journals and how to provide and store saliva samples. Once informed consent was received, subjects were then asked to complete a lifestyles questionnaire in order to get a clear idea on individual habits (number of cigarettes smoked, amount of weekly exercise, job status, medications, sleep schedule and living arrangement). Subjects were also asked to provide an examination and assignment schedule to determine when subjects were going to experience stressful versus non-stressful periods. Once these periods were determined, subjects were given an exact schedule of when to provide saliva samples, so that samples could be provided on "stressful" and "non-stressful" days. For most subjects, "stressful" days occurred on consecutive days (for example during an examination period). For a more detailed analysis, "stressful periods" were further broken down into the following periods: "pre-stress" which was the day before a stressful day or days, "stress" which was the day (s) where academic stress occurred, "post-stress' which was the day immediately following the stressful day (s) and finally "non-stressful" day(s) which corresponded to any day which was not classified in any of the previous ways. Subjects were asked to fill in the Student Life Stress Inventory (SLSI) (Gadzella, 1994) to get an assessment of how

subjects perceive of themselves during stress and their particular coping strategies. This questionnaire was scored and used at a later date to be correlated with the actual emotional and hormonal responses to stress. At the end of this preliminary meeting, a kit was given to subjects containing written instructions, sufficient filters for 30 days of saliva sampling, plastic bags to store used filters in their home freezer, numerous pairs of gloves for handling the filters, a booklet that subjects used to record their time of sampling each day, the contents of the meals they ate, their daily activities, their emotional status at the time of sampling, and detailed accounts of any medications they took on a particular day, or any acute illness suffered during the 30 days.

Salivary Cortisol (30 days)

We chose to monitor cortisol levels in saliva because subjects completed this study in their homes over the course of 30 days. It has been shown that measuring cortisol in saliva provides a reliable measure of the free unbound fraction of cortisol and correlation coefficients on the order of r=0.96 between cortisol in saliva and serum have been obtained in the elderly population (Tunn et al., 1992).

Filters (3.5 x 5 cm) were cut from Whatman no. 42 filter paper (Whatman, Clifton, NJ) for the collection of saliva. The top centimetre of the 5-cm length was used for recording subject data and was the only portion of the filter that subjects were instructed to handle (to avoid any contamination). This portion was demarcated with a small cut in the filter. The subjects were asked to place the filter paper in their mouths until the saliva front reached just beyond the 4 cm line. The filter was then air-dried and stored at -4C. In previous work with this procedure, we have established that protein content, measured in 0.1-N NaOH extracts, in salivary samples collected in this manner varies by an average of less than 1% across a wide range of samples.

Cortisol was extracted from the filter in 2mL ethanol for 1 hour at room temperature. A 300 µl aliquot of the extract was assayed using [^{125}I] cortisol as radiotracer

and a highly specific antibody (rabbit anti-cortisol, Diagnostic Systems Labs Kit). This antibody cross-reacts less than 0.61% with deoxycorticosterone, less than 3.1% with deoxycortisol, and less than 0.5% with any other adrenal steroid. Tubes were incubated at 37 degrees for one hour, centrifuged and decanted. Tubes were counted in a gamma counter with intra- and inter-assay coefficients of variation of 3.5% and 5.0% respectively.

Saliva Sampling

Over the course of 30 days, subjects were asked to provide saliva samples at four times during the day and evening: once at 8:00 am, 12:00 noon, 4:00 pm and 8:00 pm. These times were chosen to include the diurnal rhythm in secretion of cortisol. The thirty days did not have to be consecutive, but could span over two months. Subjects were asked to record the date and time of sampling in their diaries and on the filter papers and were not obligated to give their samples at the proposed time. They were told to use these times as a guide. Subjects stored their air-dried samples in their home freezers. While it is best to store saliva at temperatures lower than freezing, saliva samples can be stored at 20^0C for up to 4 weeks without significant reduction in cortisol levels (Kirscbaum and Hellhammer, 1994). Subjects delivered the samples on ice packs upon completion of the study.

Dietary Analyses

Subjects were asked to record the contents of their meals and snacks in as much detail as possible for 30 days. This included specifying quantities, how food items were prepared, name brands of foods, when food was consumed in a restaurant (including the name of the restaurant), the time of the meal, if meals were skipped and why, and the actual description of the foods eaten. There were a number of reasons for selecting this method of collecting dietary data. First, we needed a long-term and naturalistic assessment of dietary patterns in our subjects with the least amount of intrusion as possible. Since we were interested in monitoring numerous variables at the same time (saliva F, diet and affect) over a prolonged period of time and in as natural a setting as possible, any other form of

dietary assessment, such as 24-hour recall (subjects would have to be telephoned daily and asked to recall what they ate) or food frequency questionnaires (which necessitate subjects to describe how often foods are eaten) would be intrusive and potentially disturbing and stressful. The majority of subjects provided very detailed information on the contents of their meals and the detail did not vary from one day to the next. McCargar et al. (1993) found very good agreement between 3-day and 7-day food records for estimating nutrient intakes and extremely high agreement between 2 consecutive three-day records. Dwyer (1994), in reviewing dietary assessment, concluded that the diet diaries are the only method for long-term at-home diet assessment. Contents of the meals were entered into the "Foodsmart" dietary analysis program (Sasquatch software, Vancouver, BC) which breaks foods down into: grams and mg of carbohydrate, protein, fat (including mono and poly saturates and unsaturates), alcohol, calories and cholesterol.

Affective Status

Each time a subject gave a saliva sample, he/she was asked to record his/her emotions at the time. Subjects were given a list of adjectives at every time point and asked to circle the adjective(s) that best described their emotional status at that time. The list of words contained both positive (ex; "happy, excited, etc..") and negative ("sad", "depressed", etc...) words. The number of adjectives was summed and averaged. Please see the appendix for the complete diary, including instructions, dietary sheets and adjective lists.

Daily Activity

Subjects would also record their daily activities (number of classes, examination taken, assignment handed in, oral presentation, exercise, whether they worked at their job, personal details, and any other major event) and whether they took any medication (including contraceptives) that day. Females were also instructed to indicate the start and end of their menstrual cycle.

Statistics

Data were analyzed with factorial and repeated measures analyses of variance and by paired and unpaired Student's t-tests. Scheffe post-hoc tests were performed when appropriate. Integrated hormone levels were calculated using the trapezoidal rule and the data expressed over time of sampling. A median split was performed on both the mean daily saliva F and saliva F at different times in the day and subjects' whose F levels were above the median for both the mean daily F and all time of day F were designated as "high" and those below the median were designated "low". $P<0.05$ was considered significant

Results

Stress Effects- Saliva F levels (general)

Subjects showed somewhat of a diurnal pattern in salivary F at all time points that they were assessed, such that salivary F levels were highest at 8 AM (Figure 19). The one exception was during the post-stressful period, where salivary F levels were similar throughout the day. At each time period, 8 AM F levels were significantly ($p<0.05$) higher than 8 PM levels, with the exception of the post-stress period. During both the non-stress and pre-stress periods, F levels were significantly ($p<0.05$) higher at 8 AM when compared to 4 PM F levels. During the non-stress period, noon F levels were also significantly ($p<0.05$) higher than F levels at 4 PM and at 8 PM. During the pre-stress phase, 8 AM levels of F were significantly ($p<0.05$) higher than noon F levels. Finally, F levels measured at 8 AM were significantly higher ($p<0.05$) during the stress period than 8 AM levels of F measured during all other periods. F levels at noon, 4 PM and 8 PM during stressful periods did not differ from their respective levels during the other periods.

Integrated daily saliva F levels were significantly ($p<0.03$) higher during the stressful period when compared to all other periods (figure 20).

Relationships between saliva F, diet,

To assess the relationship between dietary status and saliva F, we performed regressional analyses between saliva F at all times of the day in addition to the overall mean daily saliva F level (see table 3). We found significant ($p<0.05$) and positive relationships between noon F levels and all the macronutrients measured and we consistently found a significant positive relationship between saturated fat consumption and saliva F at all times of the day (in addition to mean saliva F levels).

Saliva F- High versus Low

Based on the above relationship we confirmed that we had a population made up of individuals who had lower food consumption associated with lower saliva F (and vice versa). Figure 21 represents saliva F levels in individuals designated as "High" versus "Low" at 8 AM (figure 21 A), noon (B), 4 PM (C) and 8 PM (D) during stressful and non-stressful periods. At 8 AM, High individuals have significantly ($p<0.05$) elevated F during non-stressful and stressful periods; furthermore, individuals designated as High have significantly elevated ($p<0.05$) F at 8 AM during stress relative to their 8 AM level during non-stress. In panel B, individuals designated as High have higher F levels at noon during all time periods, and significantly higher noon F during non-stress when compared to individuals designated as Low. At 4 PM (panel C), individuals designated as High have significantly ($p<0.05$) higher F at all time periods (with the exception of post-stress, although Highs are still elevated). In Panel D, while Highs have higher F at all time periods at 8 PM, they have significantly higher F during non-stress.

Figure 22 shows the mean daily saliva F levels during stressful and non-stressful periods in individuals designated as High versus Low. Those designated High have

significantly ($p<0.05$) elevated mean daily F during both the non-stress and stress periods and elevated during pre-stress and post-stress compared to those designated as Low. High individuals have significant ($p<0.05$) elevations in mean daily F during stress compared to their levels during non-stress.

Food Consumption- High vs Low

Figure 23 shows the average daily total caloric consumption across all periods in individuals designated as High vs Low. In general, subjects consumed the same amount of total calories regardless of which period they were in; there was a decrease in caloric intake following stress ("post-stress") in the Low individuals but this was not significant. Figure 24 is daily average fat (panel A) and saturated fat (panel B) consumption during the different periods. In general those designated as High consumed more daily fat (figure 24 A) than Low individuals; during the period post-stress, High individuals consumed significantly more fat when compared to individuals designated as Low. Furthermore, Low individuals diminished their fat intake relative to what they were consuming during the other time periods, whereas High individuals' fat consumption increased steadily before, during and following stress relative to their consumption during non-stressful periods. High individuals consumed significantly more saturated fat at all time periods when compared to Low individuals (figure 24 B). Furthermore, High subjects consumed significantly more saturated fat ($p<0.05$) during stress compared to what they consumed during non-stress. Carbohydrate and protein consumption was similar in Highs versus Lows during all time periods (figure 25 A and B). Those designated as Low again reduced their consumption of protein during the post-stress periods compared to those designated as High.

In general, during all periods, subjects consumed the largest percent of their calories from carbohydrates, followed by fat and protein. During non-stress, fat, protein and carbohydrate consumption were, 28.3 %, 14.3 % and 57% respectively. During pre-

stress, % values were 28.4, 14.7 and 57% respectively, during stress, 28.5, 15.3 and 56.2 % respectively and finally during post stress, fat, protein and carbohydrate consumption was 27.1, 15.1 and 57.8% respectively. During all time periods, subjects consumed significantly ($p<0.05$) less protein when compared to both carbohydrate and fat and significantly ($p<0.05$) less fat when compared to carbohydrate. These percentages are in good agreement with what is found in the general population (Berthoud and Seeley, 2000).

Relationships between saliva F and affective status

Table 4 shows the relationship between saliva F levels during the day (and mean saliva F) and both the mean and time of day affective status of subjects. Significant positive relationships were found between 8 AM F levels and both positive and negative adjectives reported at that time of day as well as mean negative adjectives in general. Significant relationships were also found with F levels at 8 AM and positive adjectives reported noon, 4 PM and 8 PM. Noon saliva F levels were significantly related to positive adjectives reported at noon, 4 PM, 8 PM and the mean number of positive adjectives and negative adjectives reported at 8 PM. Saliva F levels measured at 4 PM were significantly related to negative adjectives reported at 8 AM and the mean number of negative adjectives; 4 PM F levels were also related to 8 PM positive adjectives. 8 PM saliva F was significantly related to negative adjectives measured at noon, 4 PM and 8 PM, and the mean number of negative adjectives reported. Finally, mean daily F was significantly related to negative and positive adjectives reported at 8 AM, negative adjectives reported at noon, positive adjectives reported at 4 PM, positive adjectives reported at 8 PM and both mean negative and positive adjectives.

Affective Status

While the number of negative adjectives remained fairly stable across all time periods, there was an increase in the mean number reported during both pre-stress and stress periods, and a decrease during the post-stress period (figure 26). There were

significantly ($p<0.03$) less positive adjectives reported during the pre-stress period when compared to the number of positive adjectives reported during stress and post-stressful periods. During the pre-stress period, there were significantly ($p<0.03$) less positive than negative adjectives reported; during the other periods, no significant differences were found between the number of negative versus positive adjectives reported. However, in general subjects reported fewer positive adjectives during stress and fewer negative adjectives during the post-stress period and similar number of positive and negative adjectives during non-stressful periods.

We assessed whether there was a difference in affective status during different times in the day (figure 27). The mean number of positive adjectives reported was similar across the day during both the pre-stress and stress periods. However, during non-stress, subjects reported significantly ($p<0.05$) more positive adjectives at 8 PM when compared to 8 AM for that particular period. Furthermore, subjects reported significantly ($p<0.05$) more positive adjectives during the post-stress period at noon when compared to the amount reported at noon during the non-stress period. In general, subjects reported the most amount of positive adjectives during the post-stress period, with the exception of positive adjectives reported at 8 AM (which was constant across periods). Negative adjectives are shown in figure 28. Subjects reported significantly ($p<0.05$) more negative adjectives at 4 PM during non-stress when compared to negative adjectives reported at both 8 AM and 8 PM. Further, the number of negative adjectives reported during stress at 8 PM was significantly higher than the number reported during non-stress at 8 PM. In general, subjects reported the highest average amount of negative adjectives at all times of the day during the stressful period.

Relationships between saliva F, diet, affective status and inventory (SLSI) scores.

To assess how food and affective status were related, regressional analyses between those two variables are shown in table 5. Total fat was significantly positively

related to positive adjectives reported at noon, negative adjectives reported at 8 PM and the mean overall positive adjectives. Protein was positively related to positive adjectives reported at noon and 8 PM and the overall positive adjectives. Carbohydrate was not related to affective status. Total calories were positively related to positive adjectives at noon and 8 PM and mean positive adjectives. Saturated fat was positively associated with negative adjectives reported at 8 AM and 8 PM, positive adjectives reported at noon and 4 PM and overall positive adjectives.

Table 6 shows the association between scores on the SLSI and time of day F, mean F and mean number of positive and negative adjectives reported. There was a significant and positive association between 8 AM, 4 PM, 8 PM and mean daily saliva F levels and SLSI scores.

Gender Effects-Saliva F levels

Figure 29 represents mean daily saliva F levels assessed during different periods in males and females. In general, females had lower mean daily saliva F levels at all time points, with the exception of saliva F levels during stress, which were similar in males and females. Time of day F levels are shown in figures 30 through 33. In Figure 30, 8 AM saliva F levels were similar in males and females during non-stressful periods; however during the pre-stress and period, females showed higher levels of 8 AM F when compared to males. During the post-stress period, females had lower F levels than males. Females had significantly ($p<0.05$) elevated 8 AM F levels during stress when compared to their respective 8 AM levels during non-stress. Noon saliva F levels were consistently higher in males during all periods when compared to females; in general levels did not change prior to, in response to, or following the stress when compared to non-stressful periods (figure 31). Both males and females had the highest level of noon F during the on-stressful periods. In figure 32, again males showed higher 4 PM F levels during all periods; males and females had the highest 4 PM F levels during stress and the lowest 4 PM F levels

during post-stress. Females had significantly ($p<0.05$) higher 4 PM F levels during stress when compared to their respective 4 PM F level during post-stress. Finally, 8 PM F levels are shown in figure 33. While there were no significant effects of gender or time period on 8 PM saliva F levels, in general males had higher 8 PM saliva F levels and males F levels did not change before, during or following stress. Females had the highest 8 PM F during stress and the lowest during pre-stress.

Diet-Gender Effects

In terms of total calories, males showed a higher consumption during all periods. In Figure 34, female consumption of total calories during pre-stress was significantly ($p<0.05$) lower when compared to males at this time point. Females consumed the least total calories during pre-stress, while males consumed a similar amount of total calories throughout. In general males consumed more total daily grams (data not shown) at each time point and significantly more during the pre-stress period ($p<0.03$). The pre-stress period caused a reduction in total grams consumed in females, whereas males increased their total gram intake during pre-stress. Dietary fat (panel A) and cholesterol (panel B) are shown in figure 35. There were no significant differences in dietary fat consumption at any of the time points assessed; females decreased their intake of fat during pre-stress while males diminished fat intake during post-stress. Females also did not modify their intake of cholesterol (panel B) before, during or following stress when compared to non-stressful periods. Males, however consumed more cholesterol during the post-stress period when compared to their consumption during all other periods and significantly ($p<0.05$) more than females during the post-stress period.

Carbohydrate consumption during different periods is shown in figure 36. Again, females consumed the least amount of carbohydrate during the pre-stress period, while males consumed the least during the post-stress period. Protein consumption was affected by stress (figure 37). Females ate significantly ($p<0.05$) less protein during pre-stress

when compared to males. During post-stress, males ate significantly more than females during this period males ate significantly more protein at this time point compared to what they consumed during non-stress.

Affective Status-Gender Effects

Figure 38 represents the mean number of both negative (panel A) and positive (panel B) adjectives reported by male and female subjects during different periods. In general both pre-stress and stress caused an increase in the number of negative adjectives reported by both males and females. During non-stress, however, females reported significantly ($p<0.05$) more negative adjectives when compared to males at this point. Both males and females increased the number of negative adjectives reported during pre-stress, Stress was associated with an even further increase in the number of negative adjectives reported by both genders: females and males reported significantly more negative adjectives during stress than during non-stress. Finally, during post-stress females reported significantly fewer negative adjectives compared to what they reported during stress. Males also reported fewer negative adjectives during non-stress but this was not significant. Both males and females diminished the number of positive adjectives during pre-stress when compared to what they reported during non-stress. During stress females significantly ($p<0.05$) reduced the number of positive adjectives reported when compared to what they reported during non-stress. Males reported more positive adjectives during stress and post-stress compared to both non and pre-stress periods, while females reported fewer positive adjectives during pre-stress, stress and post-stress when compared to what they reported during non-stress.

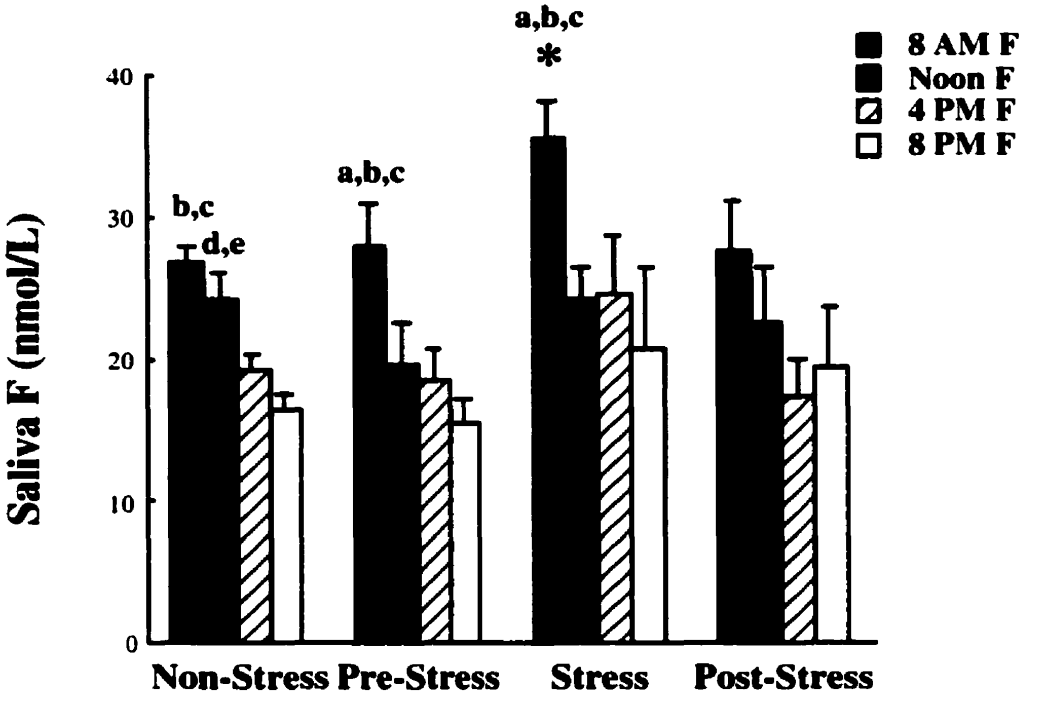

Figure 19. Mean (± SEM) saliva F (nmol/L) in subjects (n=11) immediately before, during, immediately following stressful periods and during non-stressful periods over 3 weeks. * 8 AM during stress period significantly different from 8 AM at all other periods. a 8 AM significantly different from noon at that time period; b 8 AM significantly different from 4 PM; c 8 AM significantly different from 8 PM; d noon significantly different from 4 PM; e noon significantly different from 8 PM; f 4 PM significantly different from 8 PM

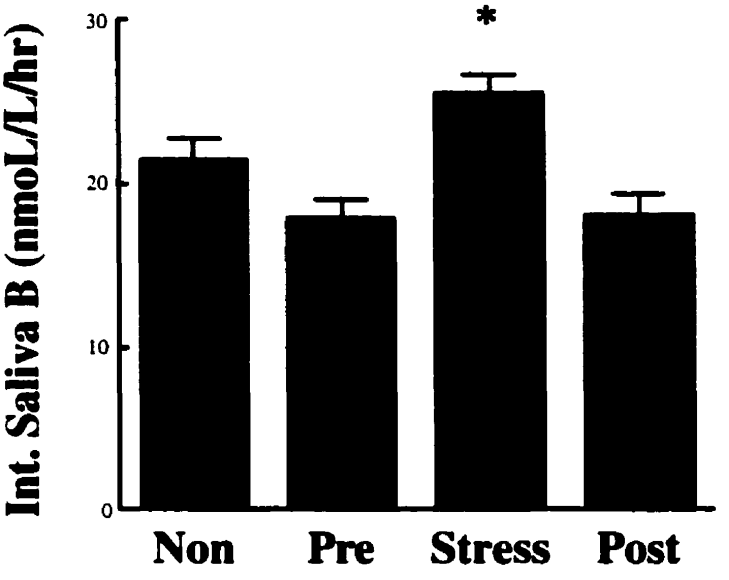

Figure 20. Mean (± SEM) integrated (nmol/l/hr) saliva F during all time periods. * stress significantly different ($p<0.03$) than all other time periods.

Table 3. Regressional analyses between time of day and mean saliva F and macronutrients

	8 AM F (nmol/l)	Noon F (nmol/l)	4 PM F (nmol/l)	8 PM F (nmol/l)	Mean daily F (nmol/l)
Total Fat (g)	0.018	0.148*	0.040	0.010	0.042
Protein (g)	0.094	0.158*	0.039	0.011	0.054
Carbohydrate (mg)	0.044	0.155*	0.002	0.045	0.032
Calories (Kcal)	0.037	0.202*	0.040	0.028	0.056
Saturated fat (g)	0.194*	0.246*	0.187*	0.192*	0.241*

*significant at $p<0.05$ or better

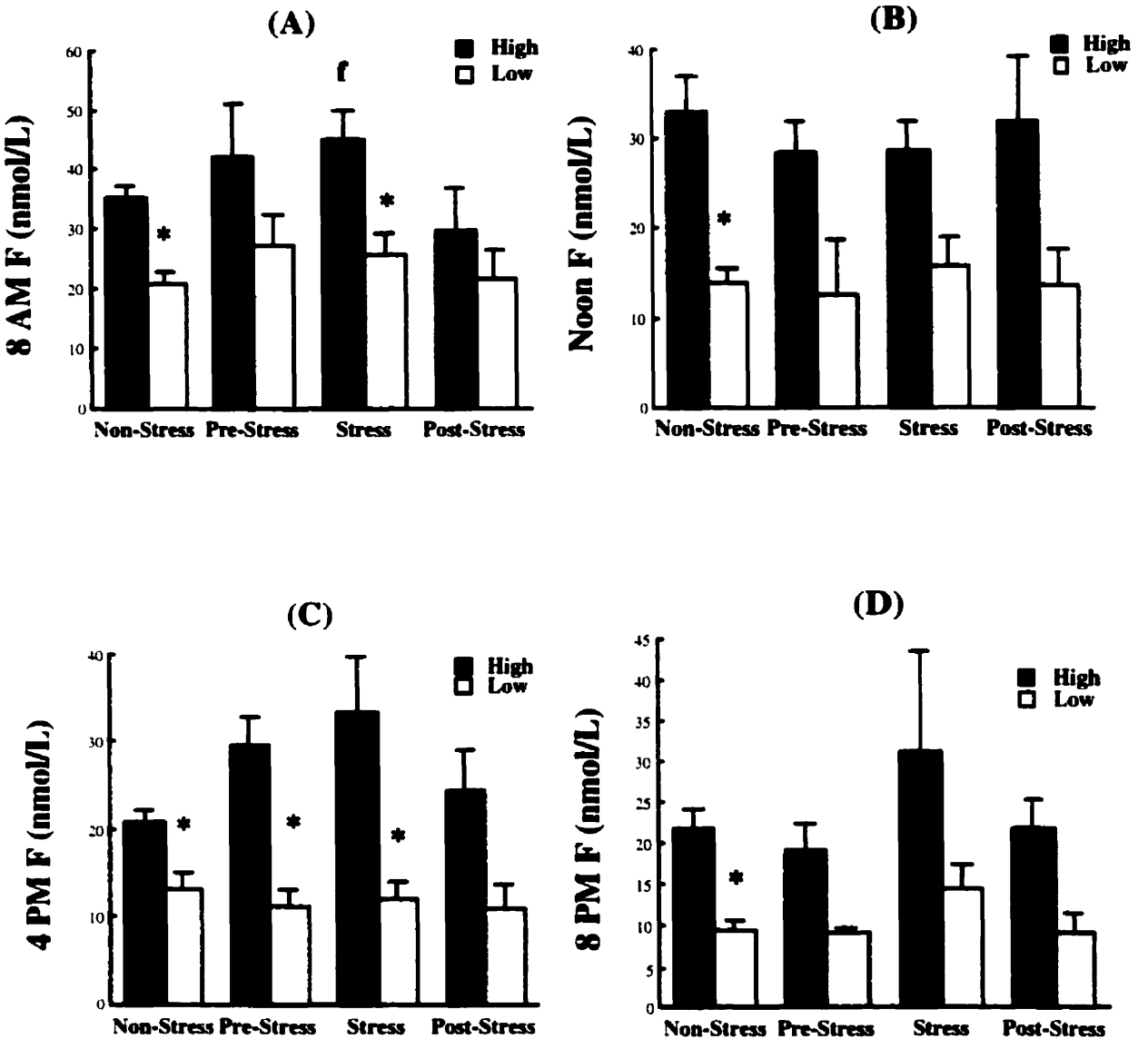

Figure 21. Mean (± SEM) saliva F (nmol/L) at 8 AM (A), noon (B), 4 PM (C) and 8 PM (D) in individuals designated as High vs Low and at stressful and non-stressful time periods. * High significantly different from Low; f stress significantly different from post-stress (within group), both at $p<0.05$.

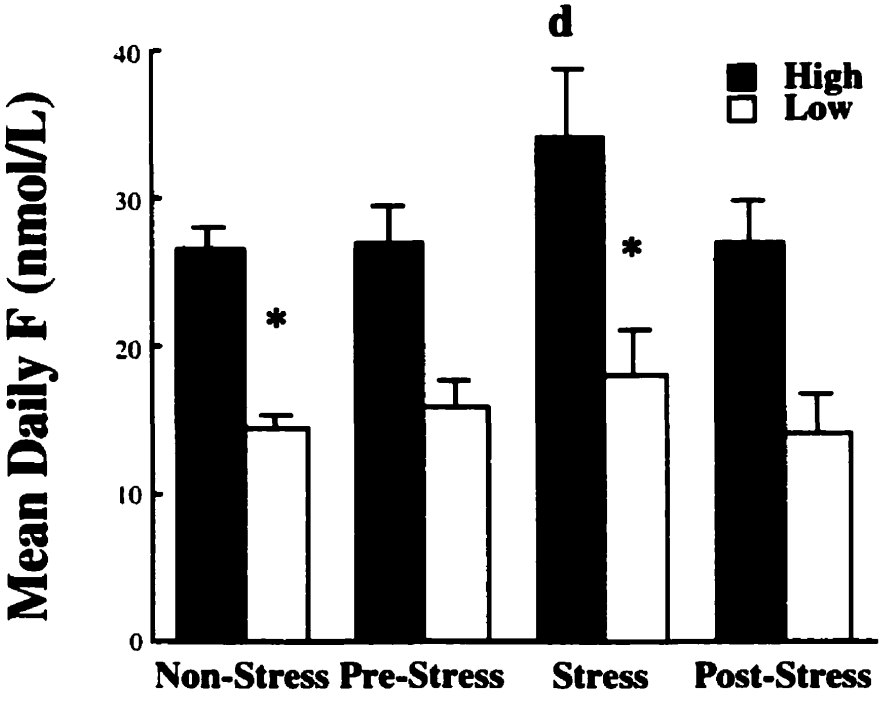

Figure 22. Mean (± SEM) saliva F (nmol/L) in individuals designated as High vs Low and at stressful and non-stressful time periods. * High significantly different from Low; d non-stress significantly different from stress (within group), both at $p<0.05$.

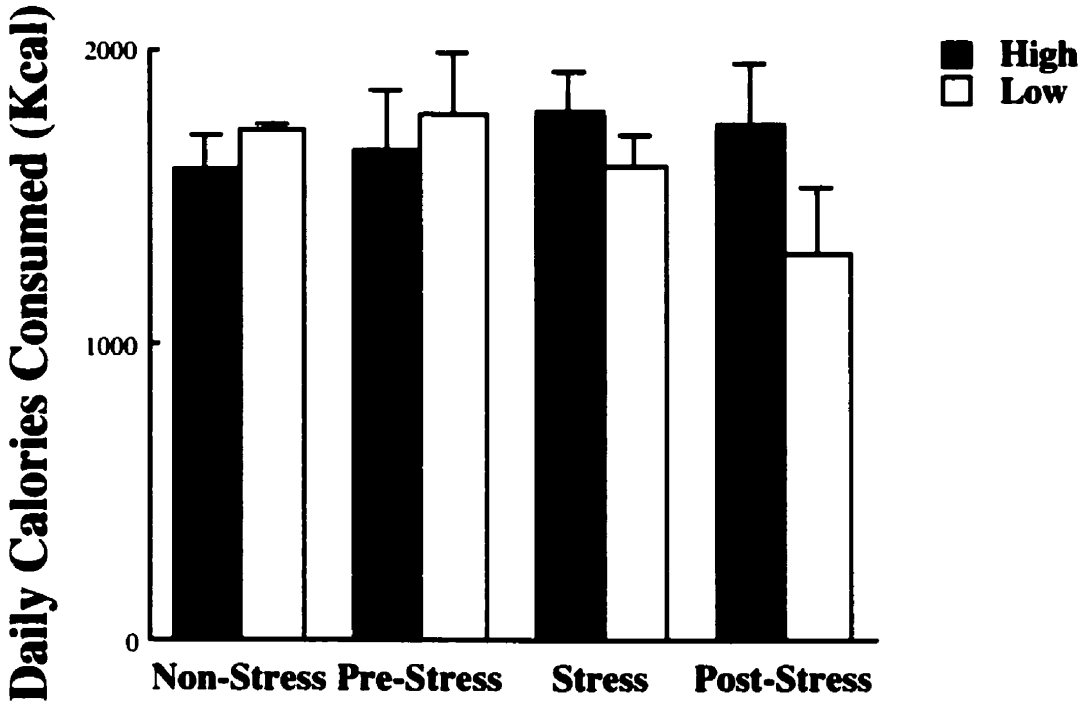

Figure 23. Mean (± SEM) daily calories (Kcal) consumed in subjects designated as High versus Low immediately before, during, immediately following stressful periods and during non-stressful periods over 3 weeks.

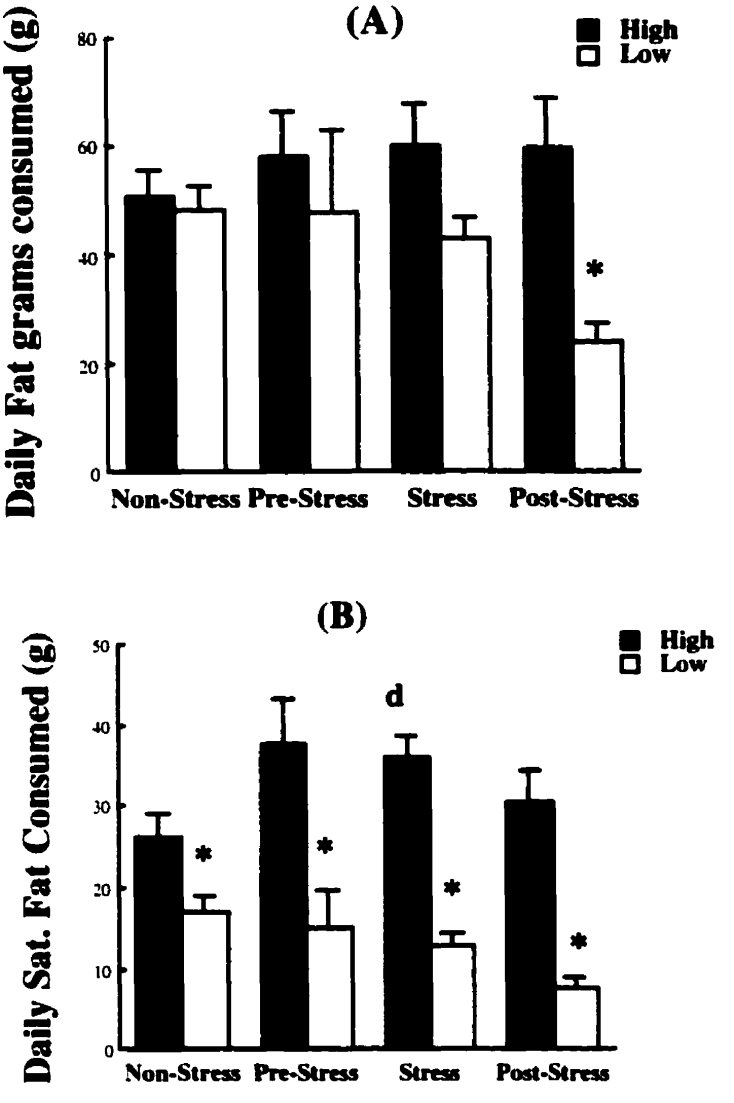

Figure 24. Mean (± SEM) daily total fat (panel A) and saturated fat grams (panel B) (g) consumed in subjects designated as High versus Low, immediately before, during, immediately following stressful periods and during non-stressful periods over 3 weeks. * High significantly different from Low; d non-stress significantly different from stress (within group), both at $p<0.05$.

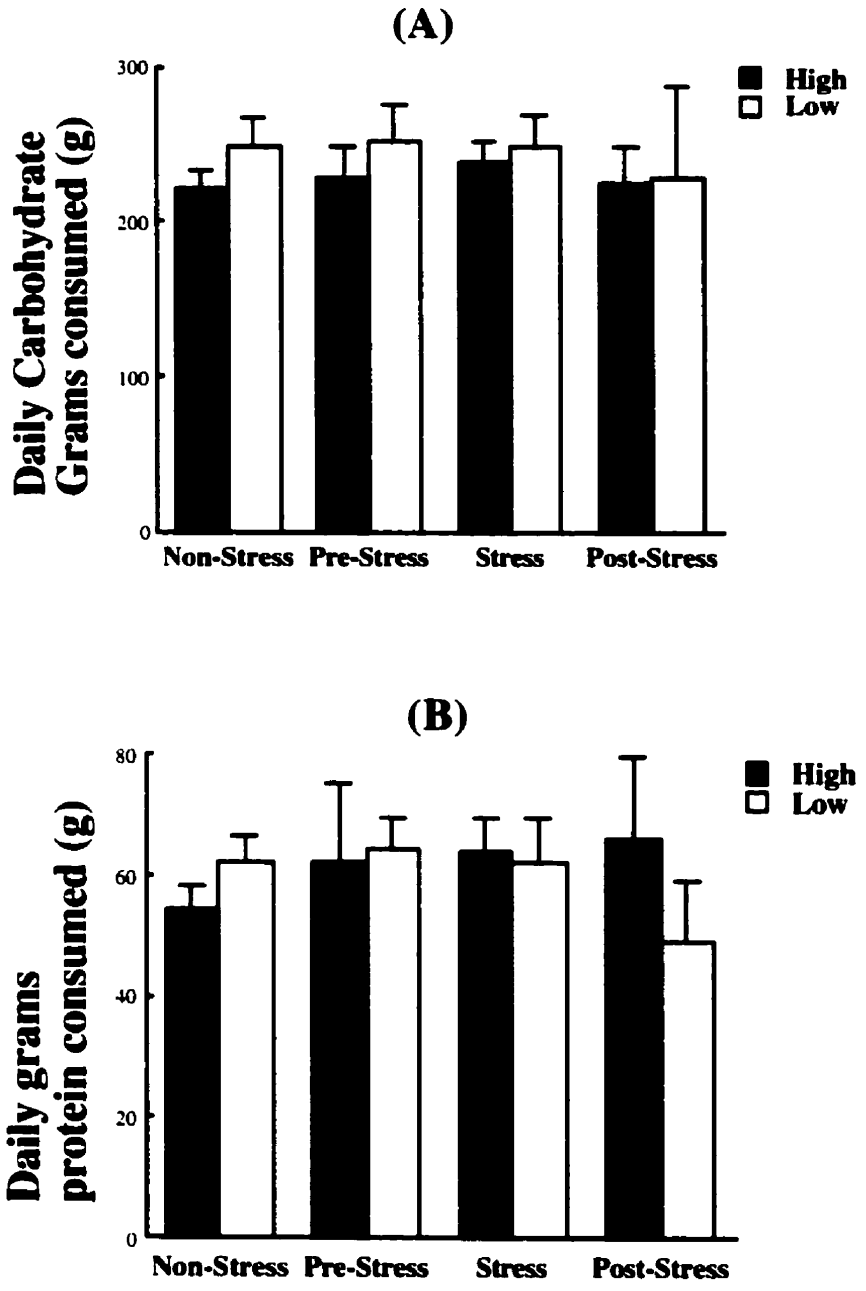

Figure 25. Mean (± SEM) daily carbohydrate consumed (g) (A) and protein (g) (B) in subjects designated as High or Low immediately before, during, immediately following stressful periods and during non-stressful periods over 3 weeks.

Table 4. Regressional analyses between time of day and mean saliva F and time of day and mean affective status (negative and positive adjectives reported)

	8 AM F (nmol/l)	Noon F (nmol/l)	4 PM F (nmol/l)	8 PM F (nmol/l)	Mean daily F (nmol/l)
8 AM negative	0.203*	0.123	0.226*	0.149	0.316*
8 AM positive	0.373*	0.127	0.100	0.055	0.186*
Noon negative	0.152	0.049	0.013	0.21*	0.159*
Noon positive	0.360*	0.267*	0.140	0.110	0.251*
4 PM negative	0.096	0.072	0.080	0.209*	0.128
4 PM positive	0.264*	0.185*	0.114	0.045	0.184*
8 PM negative	0.060	0.162*	0.025	0.149*	0.094
8 PM positive	0.254*	0.215*	0.237*	0.052	0.265*
Mean positive	0.156	0.206*	0.058	0.066	0.157*
Mean negative	0.284*	0.104	0.186*	0.229*	0.255*

*significant at $p<0.05$ or better

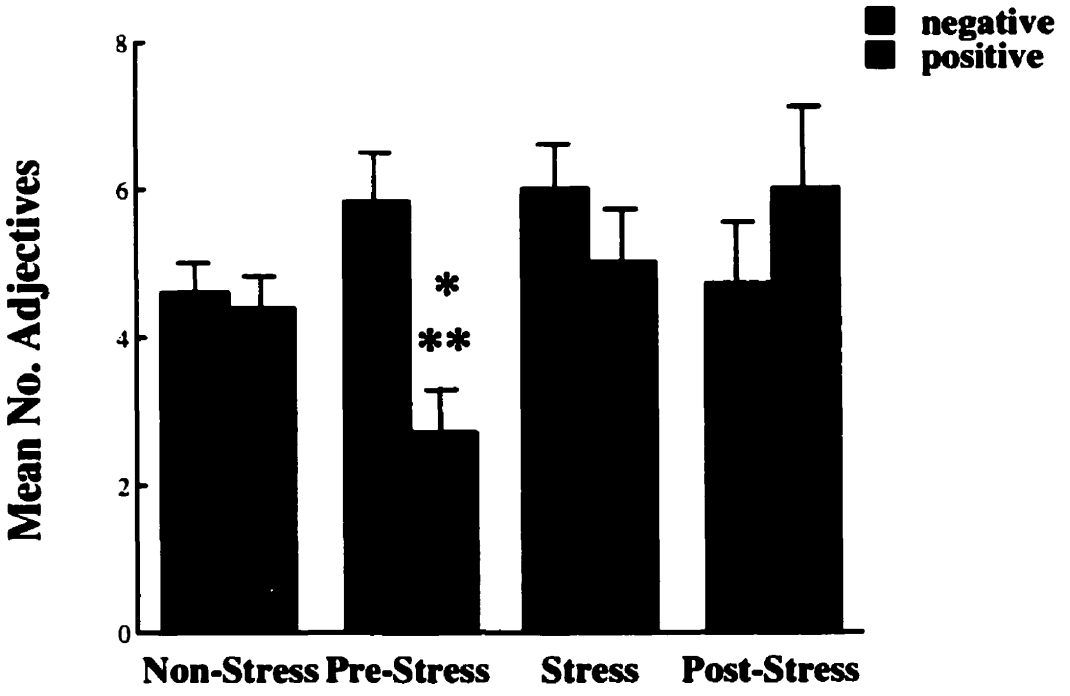

Figure 26. Mean (± SEM) number of negative and positive adjectives reported during different stressful periods. * number of pre stress positive adjectives significantly different from number of stress and post-stress positive adjectives reported; ** pre stress number of positive adjectives significantly different from number of pre-stress negative adjectives reported.

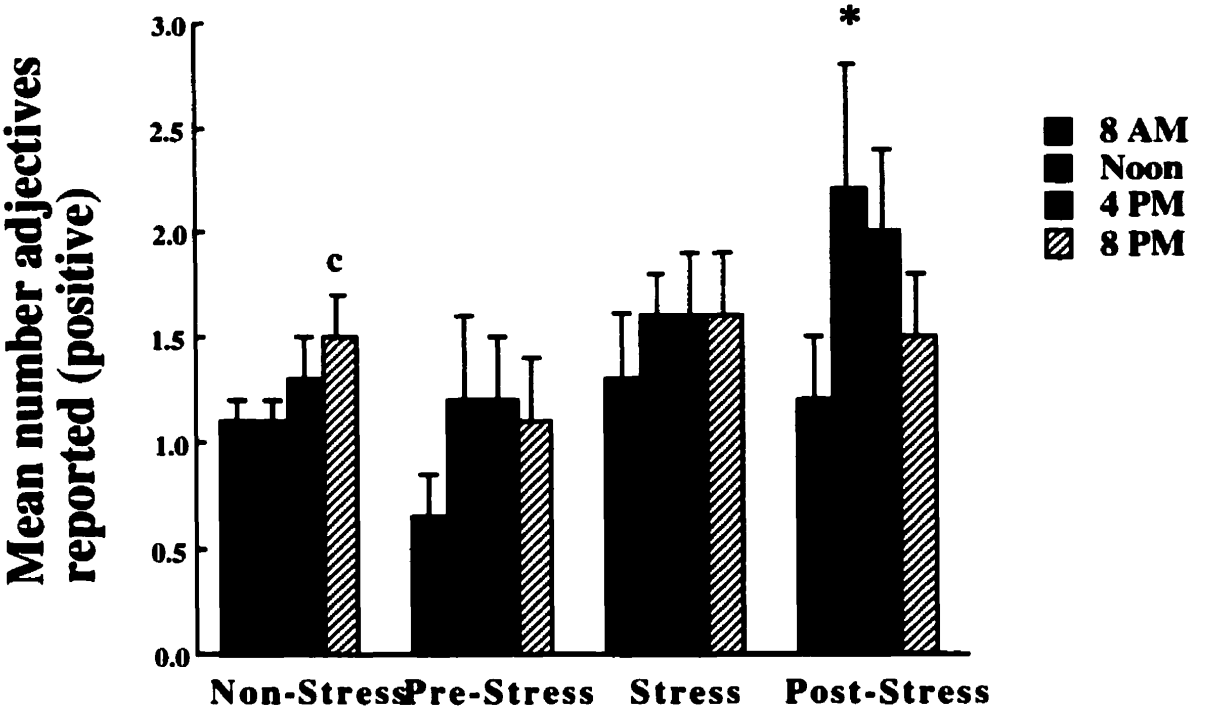

Figure 27. Mean (± SEM) number of positive adjectives reported at specific times in the day during different stressful periods. * number of non-stress positive adjectives significantly different from number post-stress positive adjectives reported.; c 8 AM significantly different from 8 PM.

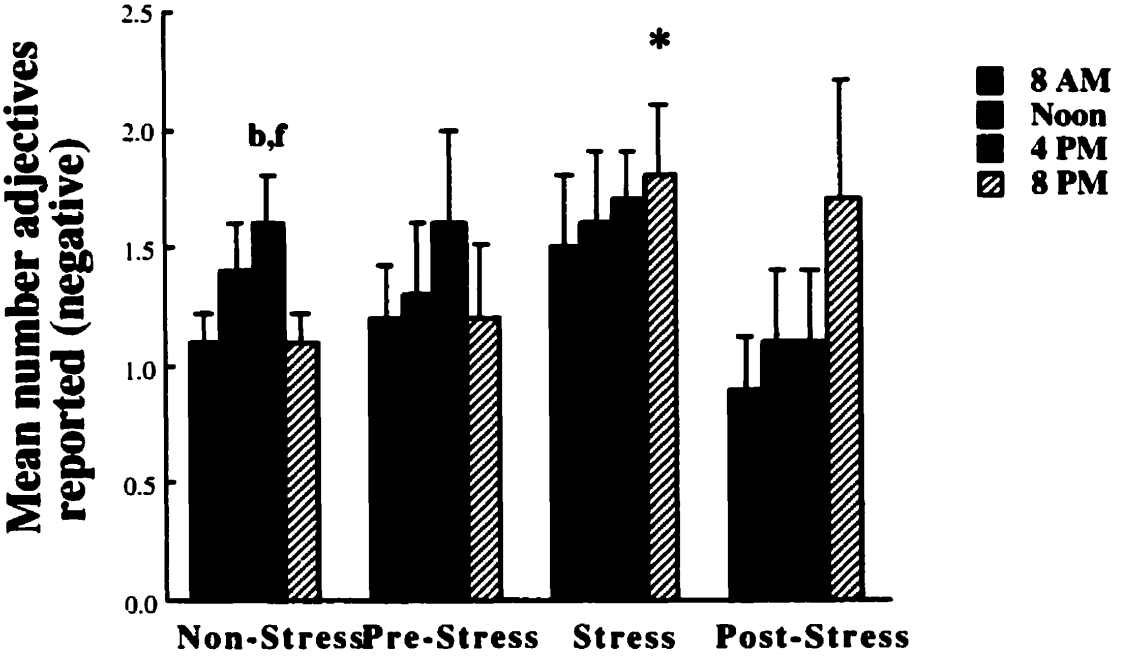

Figure 28. Mean (± SEM) number of negative adjectives reported at numerous times in the day during different stressful periods. * number of non-stress negative adjectives significantly different from number of stress negative adjectives reported; b 8 AM significantly different from 4 PM; f four PM significantly different from 8 PM.

Table 5. Regressional analyses between macronutrients and time of day and mean affective status (negative and positive adjectives reported)

	Fat (g)	Protein (g)	Carbohydrate (g)	Calories (Kcal)	Saturated Fat
8 AM negative	0.014	0.039	0.021	0.005	0.205*
8 AM positive	0.041	0.018	0.020	0.045	0.040
Noon negative	0.110	0.081	0.049	0.103	0.138
Noon positive	0.341*	0.156*	0.043	0.203*	0.285*
4 PM negative	0.047	0.076	0.063	0.023	0.084
4 PM positive	0.032	0.094	0.101	0.014	0.218*
8 PM negative	0.142*	0.005	0.038	0.044	0.269*
8 PM positive	0.091	0.160*	0.032	0.153*	0.076
Mean negative	0.058	0.079	0.082	0.106	0.112
Mean positive	0.202*	0.169*	0.069	0.214*	0.236*

* significant at $p<0.05$ or better

Table 6. Regressional analyses between Student Life Stress Inventory (SLSI)Score and time of day and mean F and time of day and mean affective status (negative and positive adjectives reported).

	SLSI Score
8 AM F	0.501*
Noon F	0.476
4 PM F	0.679*
8 PM F	0.558*
Mean Daily F	0.558*
Mean Negative	0.452
Mean Positive	0.442

* significant at $p<0.05$ or better

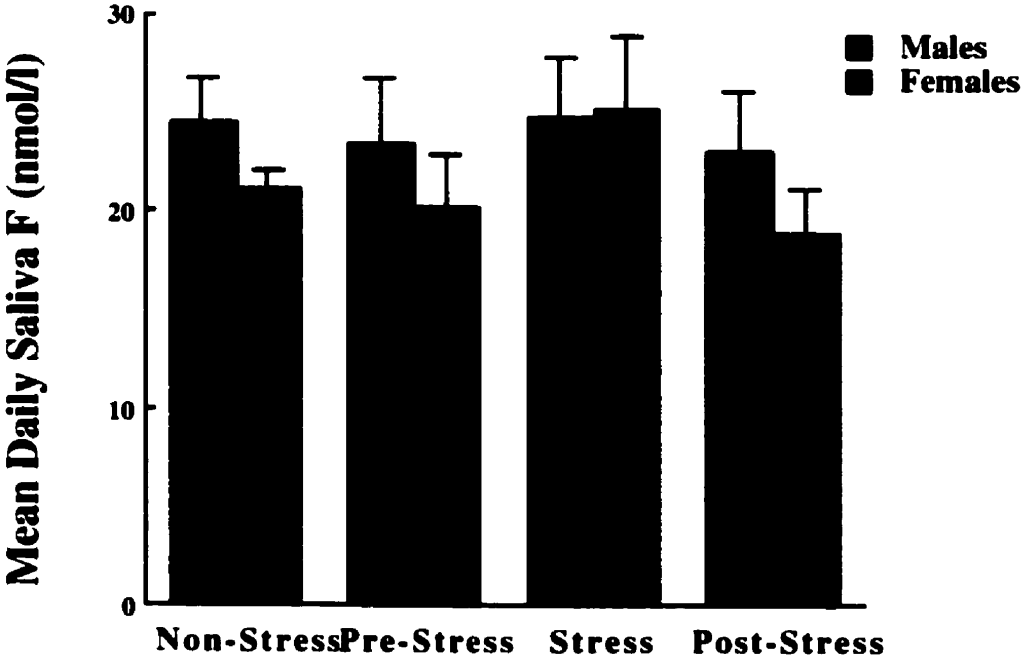

Figure 29. Mean (± SEM) daily saliva F (nmol/l) sampled at different periods over three weeks in males (n=3) versus females (n=8).

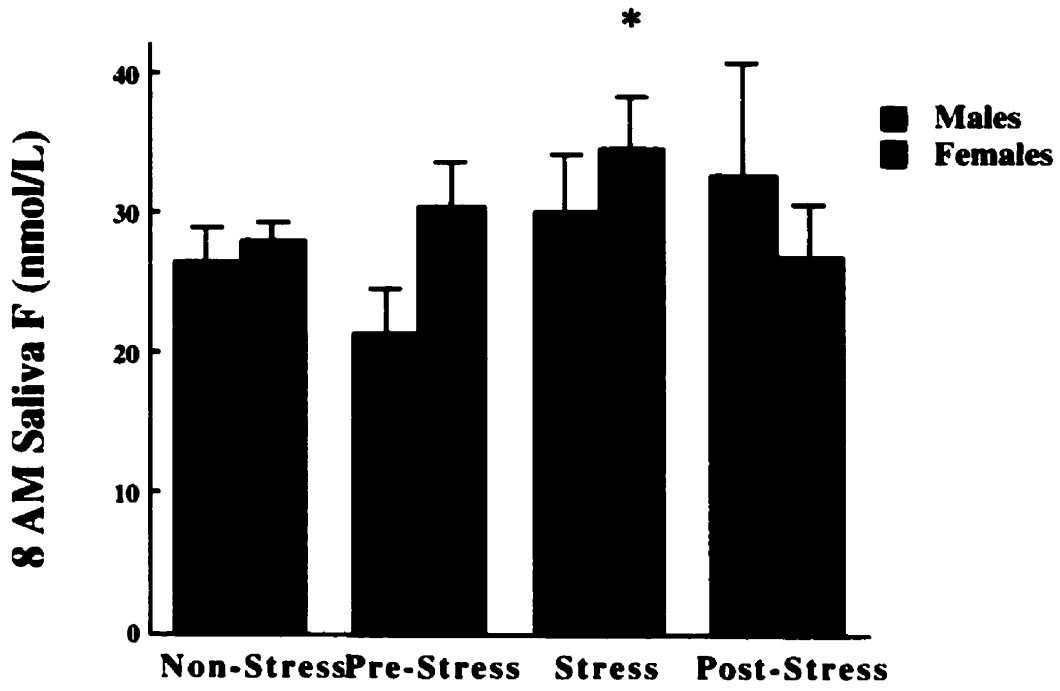

Figure 30. Mean (± SEM) saliva F (nmol/l) sampled at 8 AM at different periods over three weeks in males (n=3) and females (n=8). * female stress significantly different from female non-stress.

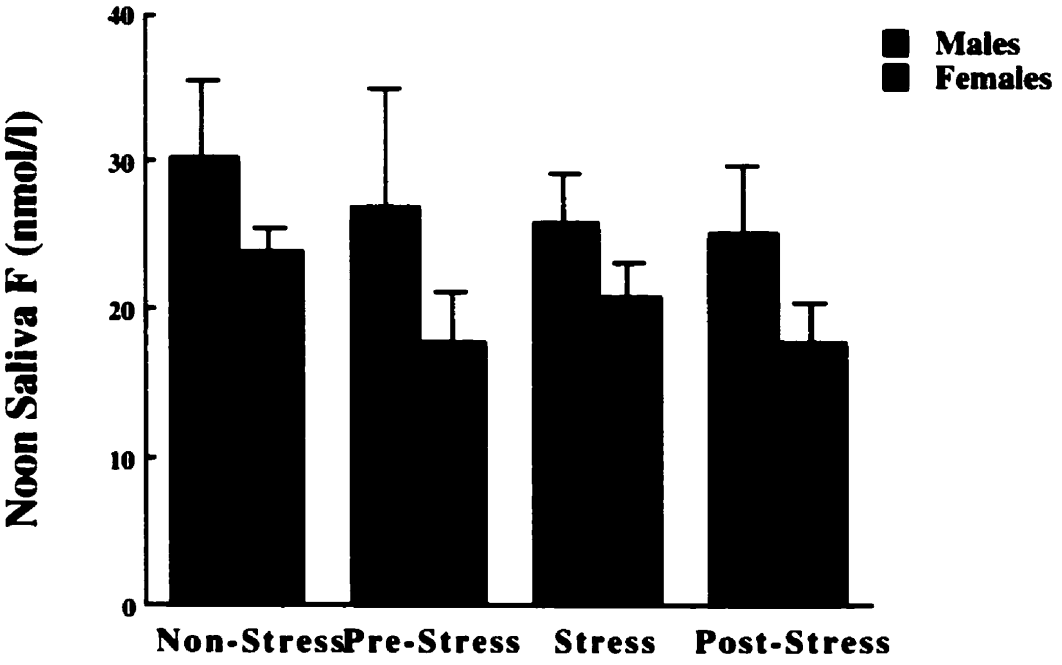

Figure 31. Mean (± SEM) saliva F (nmol/l) sampled at noon at different periods over three weeks in males (n=3) and females (n=8).

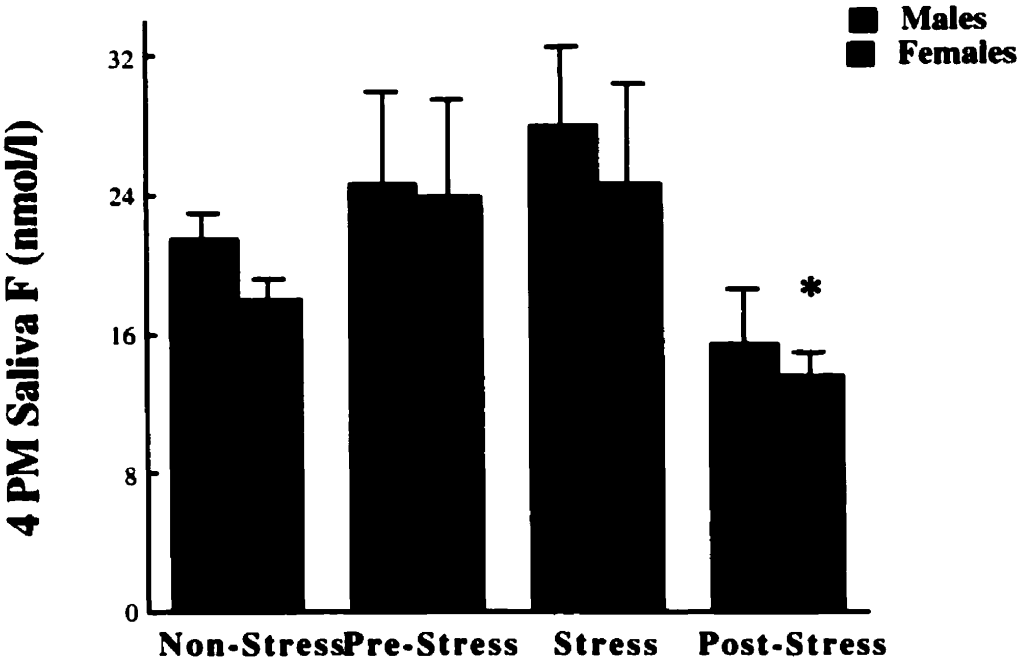

Figure 32. Mean (± SEM) saliva F (nmol/l) sampled at 4 PM at different periods over three weeks in males (n=3) and females (n=8). * female stress significantly different from female post-stress.

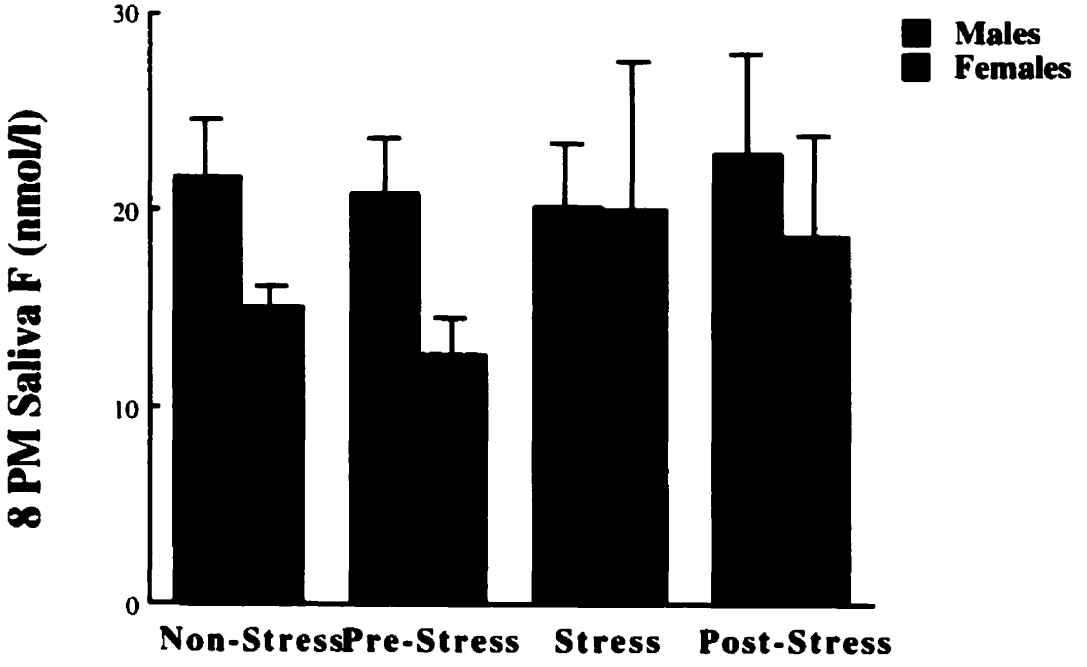

Figure 33. Mean (± SEM) saliva F (nmol/l) sampled at 8 PM at different periods over three weeks in males (n=3) and females (n=8).

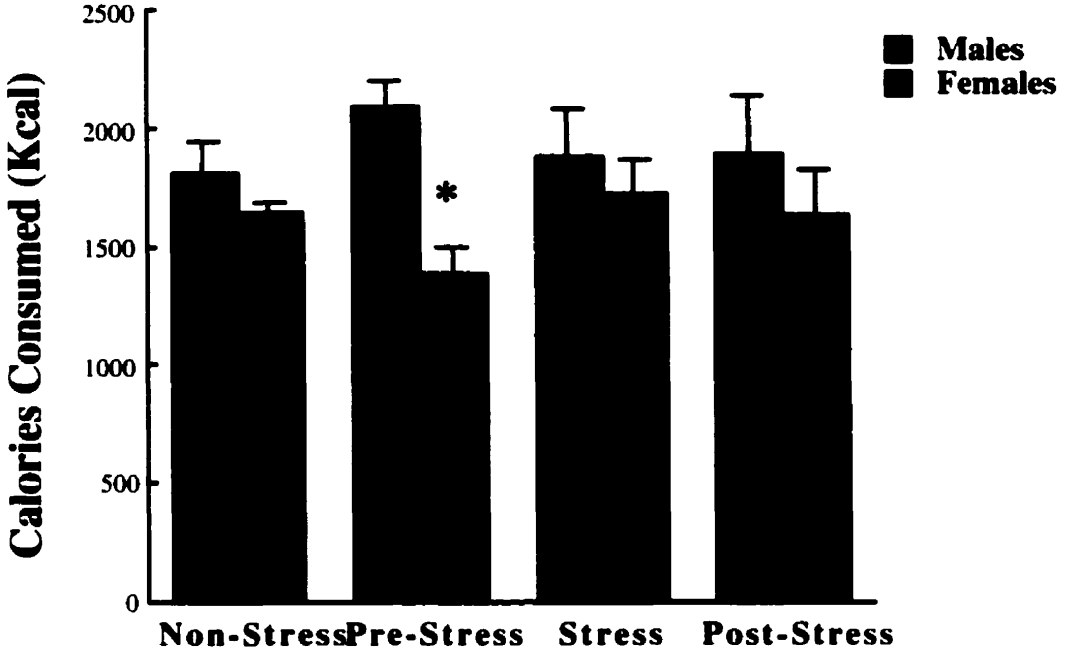

Figure 34. Mean (± SEM) daily total calories (Kcal) consumed at different periods over three weeks in males (n=3) and females (n=8). * female pre-stress significantly different from male pre-stress.

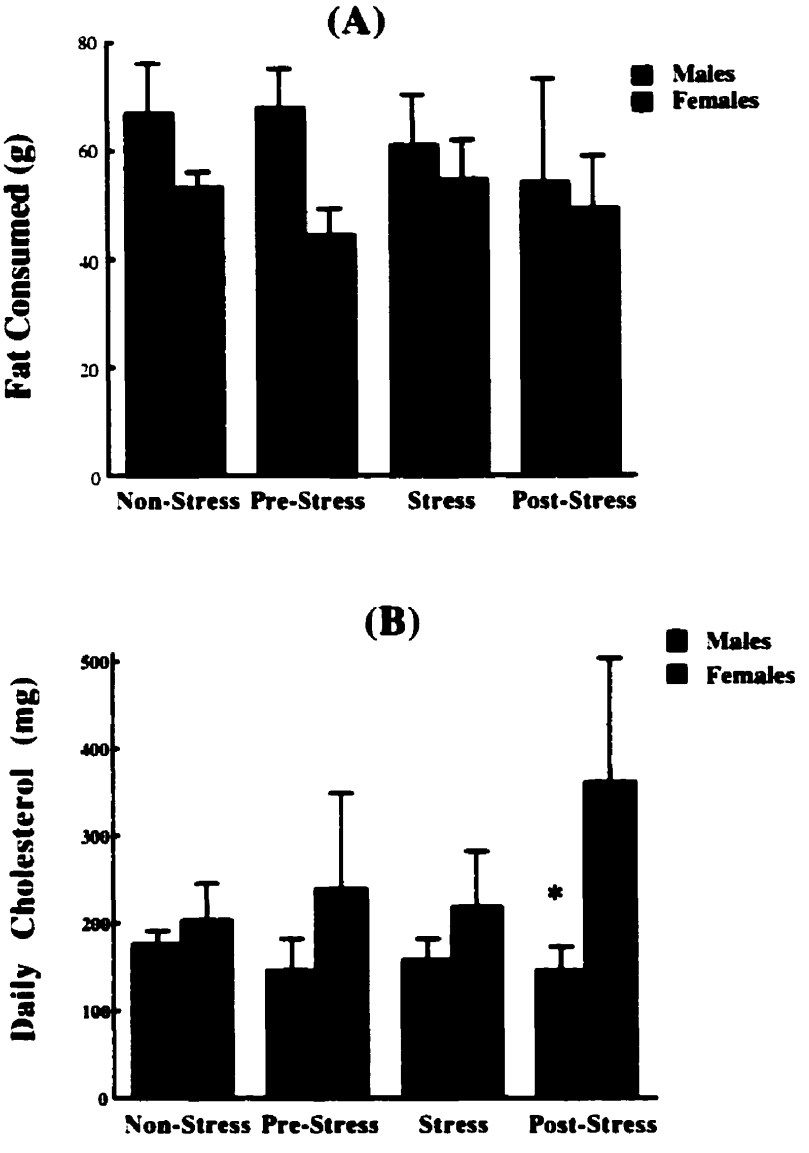

Figure 35. Mean (± SEM) daily fat grams (g) (A) and cholesterol (mg) (B) consumed at different periods over three weeks in males (n=3) and females (n=8). * male post-stress significantly different from female post-stress.

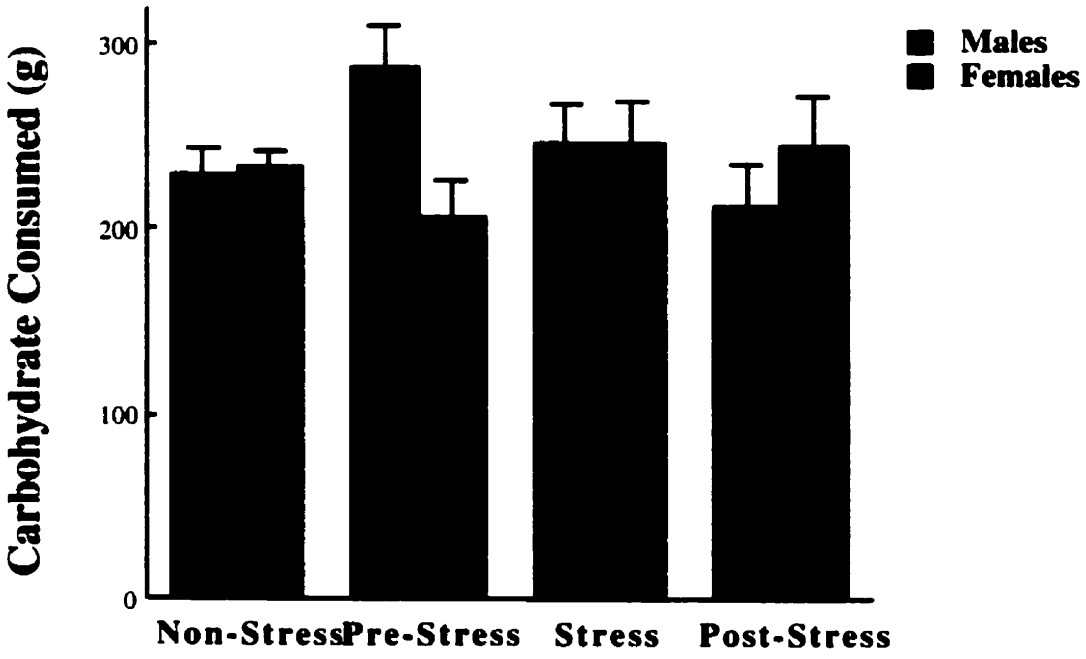

Figure 36. Mean (± SEM) daily total carbohydrate (g) consumed at different periods over three weeks in males (n=3) and females (n=8).

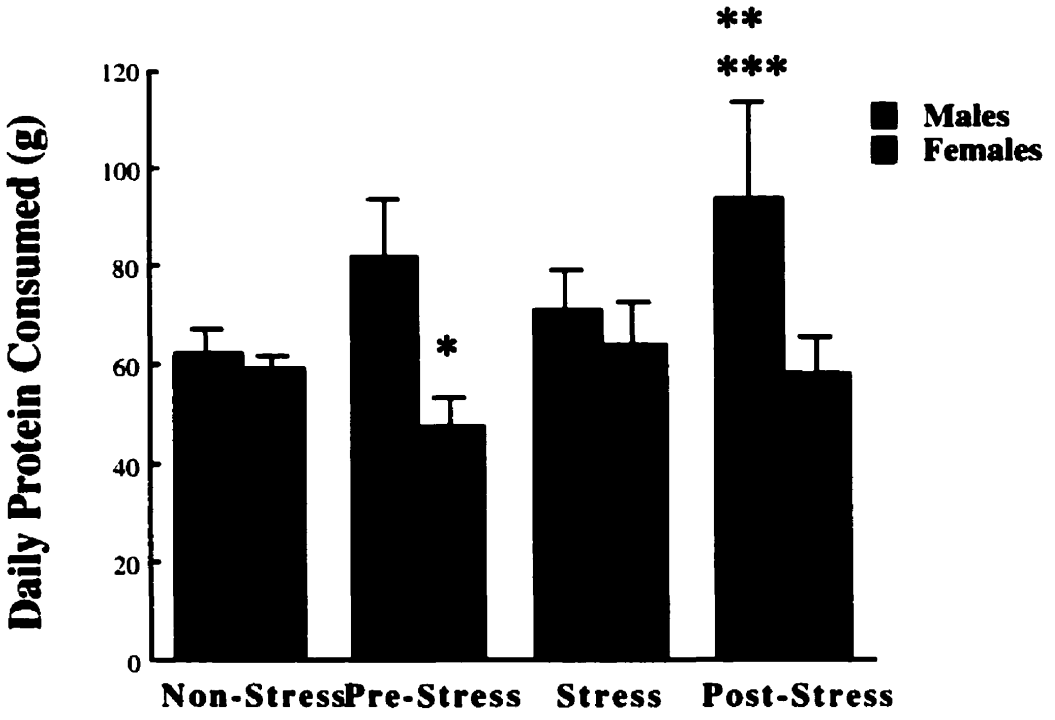

Figure 37. Mean (± SEM) daily total protein (g) consumed at different periods over three weeks in males (n=3) and females (n=8). * female pre-stress significantly different from male pre-stress; ** male non-stress significantly different from male post-stress; *** female post-stress significantly different from male post-stress.

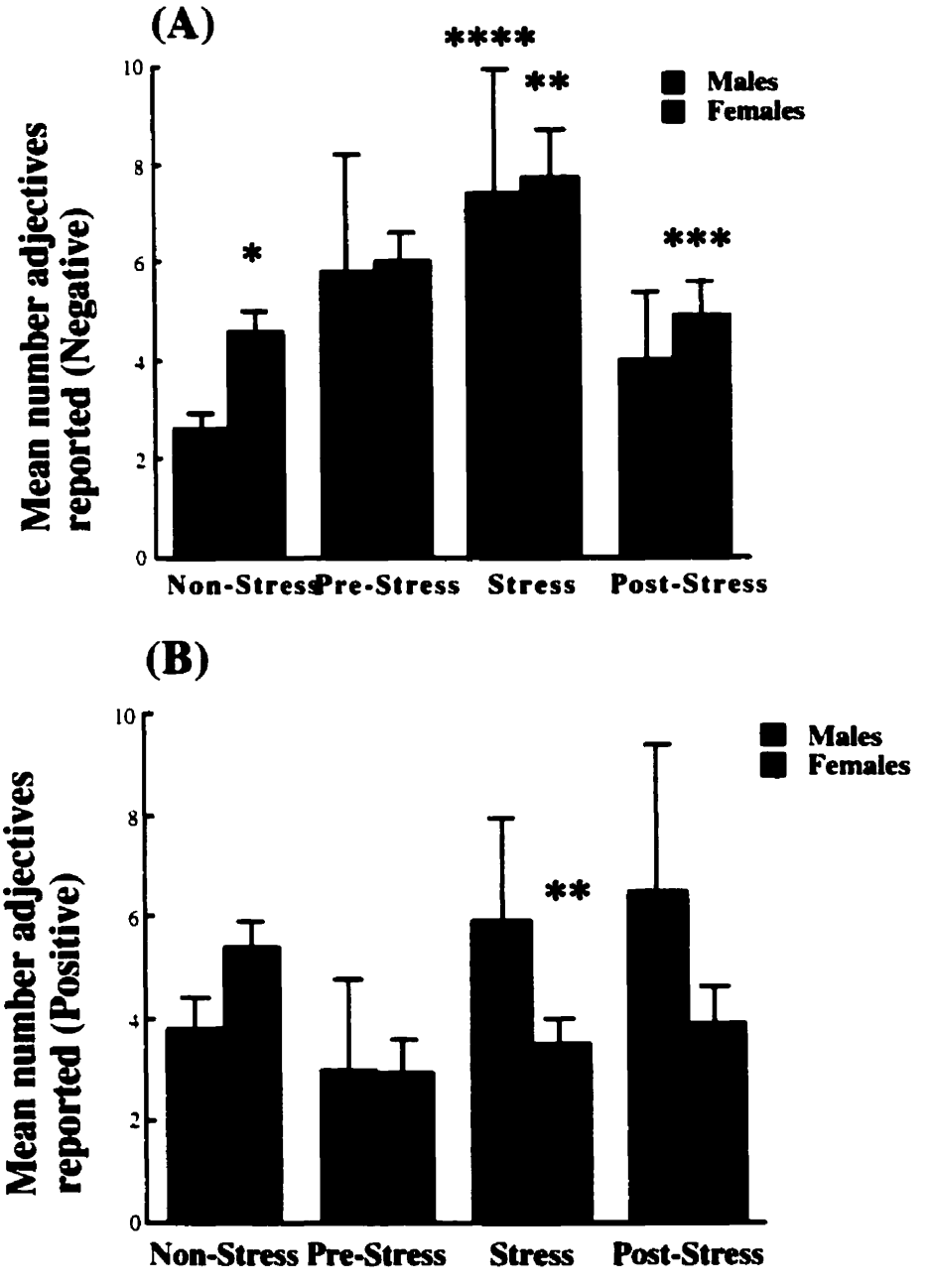

Figure 38. Mean (± SEM) daily negative (panel A) and negative (panel B) reported at different periods over three weeks in males (n=3) and females (n=8). In panel A, * female non-stress significantly different from male non-stress; ** female non-stress significantly different from female stress; *** female stress significantly different from female post-stress; ****male non-stress significantly different from male stress.

Discussion

Academic stress produced elevations in salivary F levels in subjects, with a significant elevation at 8:00 AM as well as a significant elevation in the overall mean integrated daily F level during stress. In addition, not all subjects showed an increased F response in anticipation of and during the stress, lending support for individual variation in F rhythm (Smyth et al., 1997). This finding of an increased saliva F response to stress is important for a number of reasons. First, it confirms the physiological state of the subjects, instead of relying on anecdotal or questionnaire-type assessments, confirming that academic stress, was in fact, a stressor. Second, elevations in F has been found in clinical depression; many depressed patients show a loss of the normal diurnal variation of plasma F with hypercortisolemia seen throughout the day (Sachar et al., 1973; Murphy, 1991; Nemeroff et al., 1992). In our study, not only did subjects show a significant elevation in saliva F at 8:00 AM during stress, but both High and Low subjects and subjects as a group showed a loss of the diurnal rhythm during the stress and post-stress periods. As a group, during stress, subjects had elevated saliva F at 8:00 AM but similar levels at noon, 4 PM and 8 PM. Post-stress levels of F were similar across the day, while non-stress and pre-stress levels showed a diurnal variation. Another possible explanation for elevations in AM F levels is that in general, most subjects confronted their particular academic stress during the day (ie between 8:00AM and 5PM) so exhibiting elevations in AM F levels during stress is not surprising given the anticipation of the stressor later in their day. While its impossible to confirm whether our subjects were clinically depressed it is intriguing to find that the hormonal response to academic stress shares some common characteristics with the symptomatology of depression. When we further broke our subjects down into High and Low, only high subjects showed an increased F response to stress at numerous times of day whereas our Low responders did not show an increased F response to stress at any time of day or even in their mean daily F output.

Diet was significantly affected by academic stress, and increased consumption of fats was specific to those individuals designated as High F secretors. High subjects consumed more saturated fat during stress than what they consumed during non-stressful periods and generally consumed more total fat and saturated fat when compared to Low individuals. Low responders diminished their fat intake post-stress. This finding is not surprising given the relationship between F and fat consumption. A number of groups have convincingly shown that both basal and stress-induced F or B levels are altered following high-fat diets. Carroll and Noble (1952) first demonstrated that feeding rape oil to rats caused an increase in stimulated B levels in rats, as early as after 3 weeks of feeding. Hulsmann (1978) and Brindley et al. (1981) reported similar findings. Pascoe et al. (1991) found that 3 weeks of feeding rats a high-fat diet caused an increased B and glucose response to a swim stress. Tannenbaum et al. (1997; see chapter 3 and 4) found that basal B, glucose and free fatty acid (FFA) levels were affected by as little as 7 days of high-fat feeding; ACTH and B responses were stress were also elevated. Widmaier et al. (1992) demonstrated that direct FFA infusion via the jugular vein increased plasma B in rats for up to 2 hours following the infusion. Finally, Kamara et al. (1998) showed that rats fed high-fat diets show an increased and prolonged plasma B response to an acute stress. The reciprocal is true as well: in ADX-ed rats, the daily ingestion of fat is decreased by approximately 30%, lending support for the involvement of glucocorticoids in the consumption of fat. Increased F production is related to increased preference for and intake of fat, which has been observed in obese humans (Horber et al., 1986; Castonguay, 1991). This relationship between total/saturated fat and F was specific: protein and carbohydrate consumption were similar in High versus Low F individuals.

There was a common trend for calorie and macronutrient consumption to be reduced following stress (during the post-stress days), especially in the Low individuals. Many investigators have found that food intake is dramatically decreased during and /or following acute and chronic stress in rats. Acute and chronic periods of restraint have been

shown to reduce food intake and body weight gain in rats (Kennet et al, 1985 a,b; Krahn et al., 1986; Shibasaki et al., 1988; Marti et al., 1994) and chronic intermittent footshock in rats can reduce food intake as well (Kant and Bauman, 1993). One of the potential central mechanisms involved in this stress-induced decreased in food intake is CRF. A number of animal studies have shown that exogenously administered CRF causes a decrease in food intake (Morley and Levine, 1982; Shibasaki et al., 1988; Krahn et al.,1990; Rivest and Richard, 1990; Hotta et al., 1991). Neuroendocrine activation seen during stress is driven by CRF. Thus, one consequence of stress-induced CRF activation is a reduction in food intake. Another potential mechanism for a reduction in food intake is that these acute or chronic mild stressors reduce the hedonic impact of a reward, such as food (Wilner, 1997). In fact, when we assessed the number of negative versus positive adjectives reported, it was clear that subjects reported significantly more negative adjectives during the pre-stress days and significantly less positive adjectives during the pre-stress days than during stress and post-stress. Therefore negative affect may have preceded the stress, which may be associated with a decrease in a pleasurable activity that followed the stress, such as eating. Subjects' F levels at 8 AM, 4 PM and 8 PM were correlated with the reporting of negative adjectives, suggesting a negative affective state when F levels are high.

Interestingly, the positive relationship between subjects' scores on the SLSI and their time of day and mean levels of saliva F was significant. The inventory focuses on five types of stressors that are relevant to students (frustrations, conflicts, pressures, changes and self-imposed) and four reactions to stressors (physiological, emotional, behavioral and cognitive). The higher the score on the SLSI, the greater the response to stress. Subjects were able to predict how they would respond to academic stress. This measure is critical because it allows for the assumption that our results would be reliable and replicable. It confirms that our subject's hormonal, dietary and affective responses before, during and after the stressor are accurate and reproducible.

There was, however, a significant positive association between saliva F levels at noon and all macronutrients and total calories in general. This raises an interesting issue. First, it is well known that cortisol regulates food intake and body weight (King et al., 1996; Dallman et al., 1994) and in particular has a strong influence over both the anticipation of and the consumption of total calories, carbohydrate and fat (Tempel and Leibowitz, 1994). Based upon the food records that were provided, the student population seemed to consume most of their calories during the lunch hour, making lunch their largest meal of the day. This was because many students had classes or jobs in the evening which did not allow for a meal until much after 8 PM. Therefore there are two possibilities: first, there was a putative anticipatory rise in F around noon, prior to consuming lunch, or second, there was a sustained rise in F levels upon consumption of lunch. Interestingly, saturated fat was positively associated with saliva F at all times of the day and with mean daily saliva F. While carbohydrate consumption is associated with the diurnal peak in F (prior to awakening in humans and the active cycle in rats), fat consumption occurs throughout the entire cycle (Tempel and Leibowitz, 1994).

The relationship between food consumption and affective status was also examined. In general, the consumption of fat, protein, saturated fat and total calories was associated with the mean number of positive adjectives reported. Some recent literature suggests that certain diet-induced changes in brain neurotransmitter precursors may have a functional significance. For example, tryptophan, the serotonin precursor, can increase the rate of serotonin synthesis (Carboni et al., 1989). Low levels of serotonin have been associated with depression and many current antidepressive treatments act by potentiating serotonin function (Young, 1993). A number of rodent studies have found that meals that are high in protein and/or carbohydrate can affect brain serotonin in the rat (Young, 1996) by raising levels of tryptophan (Glaeser et al., 1983). Wells et al. (1997) found that consumption of a high-fat, low carbohydrate meal induced greater feelings of sleepiness when compared to a low fat, high carbohydrate meal, suggesting a sedative action of fat. Another study found

that supplementing omega 3 fatty acids with regular pharmacotherapy to subjects with bipolar disorder prolonged the remission time; the fatty acids are purported to inhibit neuronal signaling. Low cholesterol levels have been associated with nonillness mortality (suicide or accidents) suggesting an alteration in mood that may accompany low or reduced cholesterol (Kaplan et al., 1997). Wells et al. (1997) found that lowering the fat content in the diet increased subjective ratings of tension, anxiety and stress. A recent report by Macht (1996) showed that consumption of meals that are low in total energy prior to receiving a noise stress resulted in subjects' reported more negative emotions associated with the stress.

Male subjects had higher mean saliva F when compared to females during non-stressful, pre-stress and post-stressful times; mean levels were similar during stress. When F levels were further broken down for each time in the day before, during, following stress and during non-stress, we found that only levels of saliva F in females were significantly altered by stress. While males showed an increase in 8 AM levels of F during stress when compared to non-and pre-stress times, females showed a significant increase in F levels during stress when compared to their levels during non-stress. Stress had no effect on noon levels of F in both males and females, but males had consistently higher noon levels of F at all time points measured. Males also had consistently higher 4 PM F levels during all the time periods that they were assessed compared to females but again, stress had no real affect on male 4 PM F levels. Females, on the other hand, had significantly decreased 4 PM F levels during post-stress when compared to their levels at 4 PM during stress. Finally, 8 PM F levels were again higher in males than in females and while stress elevated female 8 PM F levels (although not significantly) it had no real effect on male F levels. In accordance with our results during non-stress a number of studies have found that in general, premenopausal women have overall lower 24-hour mean basal B when compared to men (Kirschbaum et al., 1992; 1995; Born et al., 1995; Van Cauter et al., 1996; Smyth et al., 1998). There were no significant differences between males and females at any time

of day F level. Females, however, did show a significant increase in F levels at 8 AM during stress when compared to their levels during non-stress, while males' 8 AM F levels were similar during stressful and non-stressful times. Females also showed a significantly reduced F response at 4 PM during the post-stress period when compared to their 4 PM F levels during stress. Interestingly, female affective status was markedly affected by stress. During non-stress females report more positive and significantly more negative adjectives than males. While pre-stress attenuates the number of positive adjectives and elevates the number of negative adjectives equivalently in males and females, stress and post-stress dramatically decrease the number of positive adjectives reported by females only (the number that males report actually increases). Therefore one possibility is that females may differ in the cognitive/emotional processing of stressful events (Kirschbaum et al., 1992).

Dietary patterns were also affected by gender. Females significantly reduced their total calorie and protein intake before the stressful period (during pre-stress) when compared to males during this period. Again, this provides support for the notion that the anticipation of the stress is more pronounced in females than in males and corroborates the animal literature, which suggests that stress can cause a reduction in food intake. Short periods of food restriction can cause significant elevations in basal HPA activity in numerous species (Dallman et al., 1993), while stress-induced B levels remain unchanged or decrease (Akana et al, 1994). Protein restriction causes increases in both ACTH and B in chickens (Weber et al., 1990 Carcia and Weber, 1988), rats (Adlard and Smart, 1972; Mulay et al., 1982; Jacobson et al., 1997) and humans (Alleyne and Young, 1967; Smith et al., 1975) and blunts growth hormone levels in rats (Harel and Tannenbaum, 1993). Therefore one source for the emergence of elevations in the F profile in females is either a reduction in total calories or protein consumption.

Taken together these data suggest that while chronic academic stress is associated with elevations in F, not all individuals show this stress-induced elevation. Individuals

showing this elevation in F consume more fat in general and increase their consumption of fat in response to stress. This lends further support for the notion that individual differences in the response to stress may emerge as a result of or as a consequence of individual variations in dietary habits. Furthermore, affective status and diet may be related and changes in diet in response to stress may be one way individuals cope with the stressor. Finally the gender of the subject may play a role in the manner in which individuals both express and respond to stress. In sum, these data suggest that the individual variation in the response to a stressor may be subject to dietary factors which themselves modulate the processing of and coping with the stress; these habits may set a dangerous cascade in motion such that poor dietary habits during periods of stress may increase the risk for development of pathologies associated with chronically elevated GCs.

Study 3 High Fat Consumption, Basal and Stress HPA Activity and Carbohydrate Metabolism

In study 2, increased fat consumption was related to elevations in F. While associations were drawn between these two variables, a direct assessment of the effects of fat consumption on F levels was not carried out. While it is generally accepted that diets high in fat are pathological from a metabolic standpoint, the mechanism by which high-fat diets accelerate the progression of disease is relatively unknown. Knowing that a relationship exists between F levels and the consumption of fat (study 2), we wanted to explore this further by investigating the effects of high-fat feeding on both basal and stress-induced HPA functioning. Perhaps the negative effects of high-fat diets are mediated by increases in B, which then set a dangerous cascade of both central and peripheral changes in motion.

Previous work has confirmed that high-fat diets contribute to insulin resistance (Reaven et al., 1967; Storlien et al., 1986), impaired glucose metabolism (Glueck et al.,

1969), Type-2 or non-insulin-dependent diabetes mellitus (NIDDM) (Amercian Diabetes Association, 1987; Himsworth, 1935; Kolterman et al., 1979), stroke and coronary artery disease (CAD) (Lipid Research Clinics Program, 1984), although the mechanisms underlying these effects are not completely understood. Dietary cholesterol is associated with increased low-density-lipoprotein (LDL) concentrations and the elevated triglyceride levels in very low-density lipoproteins (vLDL). Dietary fat not only lowers glucose uptake, but also stimulates inappropriate glucose production (Anderson and Sieling, 1985) resulting in elevations in both circulating insulin and glucose (Glueck et al., 1969; Reaven et al., 1967). High-fat diets decrease the number of insulin receptors in liver, skeletal muscle and adipose tissue, decrease glucose uptake into skeletal muscle and adipose tissue, and decrease hepatic glycolysis and glycogen synthesis (Anderson and Sieling, 1985). Glycogen accumulation and glucose oxidation are also lower with high-fat diets and the rate of gluconeogenesis is increased in the liver (Anderson, 1982), a common problem for many diabetics. In sum, high-fat diets are associated with a Syndrome X-like state that includes hypertriglyceridemia, decreased high density lipoproteins (HDL), high LDL and vLDL, abnormal glucose production, hyperinsulinemia and insulin resistance (Reaven, 1988).

High-fat diets may also influence HPA activity, elevating adrenal glucocorticoid (GC) production (Brindley et al., 1981,Hulsmann, 1978; Pascoe et al., 1990). This is of considerable interest here since increased levels of GCs also stimulate secretion of triglycerides from the liver in vLDL, as demonstrated in perfused liver and monolayer cultures of hepatocytes (Bartlett and Gibbons, 1988; Mangiapane and Brindley, 1985). Further, GCs decrease levels of lipoprotein lipase, which controls the hydrolysis of vLDL; this decrease has been shown to exaggerate hypertriglyceridemia (Taylor and Agius, 1988). Normally, most LDL formed after the degradation of vLDL is removed from the circulation via receptor-mediated endocytosis (Goldstein and Brown, 1977). The binding and

degradation of LDL by rat hepatocytes is decreased by dexamethasone, a synthetic GC, which could result in elevations in LDL levels.

A prolonged excess in GC levels leads to various adjustments, altering the balance between insulin and GCs. Elevated GCs antagonize most of insulin's actions and result in increased basal and glucose-stimulated insulin levels and pancreatic beta cell hyperplasia (Lenzen and Bailey, 1984; Martin-Sanz et al., 1990). Insulin inhibits the secretion of triacylglycerol, phospholipid, cholesterol ester and apolipoproteins B and E associated with vLDL (Brindley et al., 1988). GCs antagonize these effects by increasing the breakdown of protein, glycogen and triacylglycerol. Amino acids that are released from proteins can be used for gluconeogenesis. Other enzymes that are released from this pathway are increased in activity by GCs. Thus, the effects of increased GCs mimic those of a high-fat diet raising the possibility that some of the effects of high fat might be mediated by increases in circulating GC levels.

In the present study, we tested the hypothesis that elevations in hypothalamic-pituitary-adrenal (HPA) activity could modulate some of the effects of high-fat feeding. To accomplish this, we have examined both basal and stress-induced alterations in HPA axis functioning, and carbohydrate and fatty acid metabolism following short- and long-term exposure to a high-fat diet. We also monitored spontaneous growth hormone (GH) secretory profiles to assess the specificity of the response, and assessed dietary-induced alterations in exogenous B negative feedback efficacy .

Methods

Animals

Due to the multi-centre nature of this study, different rat strains were used. Adult male Long-Evans hooded rats (Charles River Canada, St. Constant, Quebec) were used in most experiments. In both the negative feedback experiment (M.F. Dallman Lab, University of California at San Francisco (UCSF)) and the experiment on the effects of

high-fat diets on spontaneous basal B and GH levels (G.S. Tannenbaum Lab, McGill University) adult male Sprague-Dawley rats (Harlan-Holtzman, Madison, WI and Charles River, St.Constant, Quebec) were employed. Long-Evans rats were housed on 12-h light, 12-h dark cycle (lights on at 0800 h) and were group housed until catheterization (see below). In the negative feedback and basal B/GH studies, the rats were individually housed, upon arrival, on a 12-h light, 12-h dark cycle (lights on at 0600 h) in a humidity- and temperature- controlled environment. In the basal study, body weight was monitored daily and 24-h food intake was assessed daily over several days. The rats used in these experiments weighed 260-320 g (basal and feedback experiments) and 175-225 g (stress experiment) at the onset of the experiment, and were assigned randomly to either the groups fed the control or high-fat diets for 5 days or 1, 3, 9 or 12 weeks. A subset of these rats were implanted with indwelling jugular catheters (see below), while those selected for negative feedback and glucocorticoid receptor binding were not catheterized.

Diets

Both the control (4% fat) and high-fat (20% fat) diets were obtained from ICN Biomedical (Mississauga, Ontario, Canada). The same source of diet was used in both the McGill and UCSF studies. The diets were modified from a previous study (Brindley et al., 1981) and were modified slightly for the present studies (see Table 7). Diets were balanced for protein as a percentage of energy intake and for essential vitamins and minerals. The fat source in both the control and high-fat diets was corn oil. The high-fat diet contained 4.8 Kcal/g and the 4% fat control diet, 4.0 Kcal/g. In place of fat (corn oil), the 4% fat diet contained a slightly greater amount of cornstarch.

Basal blood sampling experiments

To assess basal levels of B and GH, chronic intracardiac venous cannulae were implanted under sodium pentobarbital anesthesia (50 mg/kg i.p.), as described previously

(Tannenbaum and Martin, 1976). After surgery, the rats were placed directly in isolation test chambers and given free access to regular Purina rat chow (Ralston-Purina, St. Louis, MO) and tap water until their body weights returned to preoperative levels. During this time (5-7 days), all rats were weighed and handled daily. At the end of the recovery period, the rats were randomly divided into a group fed the high-fat diet and a group fed the control diet. All animals were presented with the same amount of either the high-fat or control diet each day and their intake was measured the following day by subtracting uneaten food plus spillage from total food given; spillage was collected on a diaper under the rat cages. In this experiment, rats were fed the control or high-fat diet for either one or three weeks prior to testing.

On the day of testing, food was removed 1.5-2 h before the start of sampling and returned at the end. Blood samples (0.4 ml) were withdrawn every 15 min for periods of 6 hours (10:00-16:00 h). All blood samples were centrifuged immediately and the plasma was separated and stored at -200C for subsequent measure of B and GH. Red blood cells were resuspended in isotonic saline and returned to the animal after removal of the next blood sample to prevent hemodynamic instability.

Stress testing

Following 1, 9 or 12 weeks on the diets and three days prior to testing, another set of animals was anesthetized under metofane (Methoxyfluorane, MTC Pharmaceuticals, Mississauga, Ontario) and implanted with indwelling silastic jugular catheters (Dow Corning) which were led subcutaneously and externalized to the nape of the neck. The catheter was filled with heparinized (100 U/ml) isotonic saline and closed off with a stainless steel obturator. Animals were housed singly for the remaining 3 days of the study (while being maintained on the high-fat or control diets).

Restraint stress was performed between 1000 and 1300 h using tubular, plastic restrainers lined with foam rubber. This period was chosen to avoid the elevated basal B

levels and peak HPA responses to stress associated with the dark phase of the cycle (Bradbury et al., 1984). A blood sample (0.15 ml) was taken immediately before the rat was placed (termed –20 min) in the restrainer and within 10 seconds following removal from the home cage. The animals were restrained for 20 minutes and blood samples were taken at both 5 ("-5 min") and 10 ("-10 min") minutes after the onset of restraint. Additional blood samples were obtained at the termination of restraint (time "0") and at 20, 60 and 120 minutes thereafter. Blood samples for B, fatty acid (FA) and glucose measurement were collected into tubes coated with EDTA, placed on ice, and then centrifuged and stored at -20^{0}C until assayed. Blood samples for ACTH assays were collected in tubes containing EDTA and Trasylol, centrifuged and stored at -20^{0}C as well.

Basal plasma glucose and fatty acid levels

To assess basal plasma glucose and fatty acid levels, separate sets of animals (non-catheterized) were fed the high-fat diets for 1, 9, or 12 weeks. Following termination of the dietary periods, the animals were killed rapidly (i.e. < 10 sec.) by decapitation after removal from the home cage between 10:00 and 13:00 h. Trunk blood was collected and stored at -20^{0}C until assayed. Plasma FA were determined with a NEFA-C test kit (Wako, Richmond, VA,23237). The assay protocol followed the manufacturer's instructions but was miniaturized and absorbancies were measured with a microtitration test reader. Plasma glucose concentrations were measured by an automated glucose oxidase method using a Beckman Glucose Analyzer 2 (Beckman Instruments, Fullerton, CA).

Adrenalectomies, brain dissections and glucocorticoid receptor (GR) binding

At the end of 1, 9 and 12 weeks exposure to the diet, two additional groups of control and high-fat-fed rats (non-catheterized) were bilaterally adrenalectomized (ADX). These animals were sacrificed by rapid decapitation 12-14 hours following ADX, a time period that allows for clearance of the endogenous steroid (McEwen and Wallach, 1973). The

brain was removed quickly and placed on ice, and the hippocampus, frontal cortex, hypothalamus and pituitary were dissected, frozen on dry ice and stored at -80^{0}C. On the day of the GR binding assay, brain tissue was homogenized in 30 mM Tris, 1 mM EDTA and 1 mM dithiothreitol, 10% (v/v) glycerol, 10 mM sodium molybdate, (TEDGM; pH adjusted to 7.4) and the homogenates centrifuged at 40C for 45 min at 105,000 x g.

Binding in all tissues was measured by single point assays in which aliquots (225 µl) of the soluble fraction from a single animal were incubated for 18-24 h [a time that has been shown to be sufficient for maximal exchange to occur and during which binding is stable (Kalimi and Hubbard, 1983) with 150 µl of TEDGM containing a saturating, 10 nM concentration of [3H] dexamethasone (88.7 Ci/mmol; Amersham, Oakville, Ontario). Nonspecific binding was determined in parallel incubations containing a 500-fold excess of unlabeled RU28362. RU28362 binds selectively to the GR receptor, with very little affinity for the mineralocorticoid (MR) receptor (Reul and DeKloet, 1985).

Separation of bound from unbound steroid was achieved using Sephadex LH-20 (Pharmacia Fine Chemicals, Dorval, Quebec) columns of 10 x 1 cm made with disposable pipette tips filled with one 3 mm glass bead and equilibrated with TEDGM. Following incubation, 100 µl of the samples were washed onto the columns with 100 ul TEDGM. The columns were eluted 30 minutes later with 500 µl of TEGM into mini-vials, which were then filled with 4.5 ml of Liquiscint (National Diagnostics, Sommerville, NJ) and radioactivity was determined in a Packard scintillation counter at 56% efficiency. Protein content was determined by the method of Bradford (Bradford, 1976) and the results expressed as fentomoles specific binding/mg protein. Protein concentrations ranged from 300-500 µg/ml for hippocampus and frontal cortex and 150-250 µg/ml for hypothalamus and pituitary.

Insulin Sensitivity Tests

At the end of 12 weeks exposure to either the high-fat or control diets, animals were food-deprived for 12 hours overnight and blood was collected via the tail vein at 08:00 h the following day for measurement of basal plasma glucose levels (termed "pre"). Two hours later, animals were injected (i.p.) with 0.125 Units/kg of insulin (Humilin R, Eli Lilly & Co., Indianapolis, IN). Samples were then taken at 15, 30, 60, 120 and 180 minutes post injection. All samples were collected in tubes containing EDTA, centrifuged and stored at -30^{0}C until assayed for plasma glucose.

Test of feedback with exogenous B

In this study, rats were either bilaterally ADX or sham surgery and implanted with either 0%, 25% or 50% B pellets. The pellets remained implanted for five days while the rats were exposed to either the high fat or control diet. At the end of 5 days, and within 2 hours of lights on, rats were exposed to 30 minutes of restraint stress. Blood was collected immediately prior to (0 minute) and at 15 minutes into restraint. At the termination of restraint (30 minutes), animals were rapidly decapitated and trunk blood was collected at this time point as well. Trunk blood was collected into plastic tubes and the plasma separated and stored at -20^{0}C for subsequent assay of ACTH and B.

Radioimmunoassays

Plasma B was measured by the Radioimmunoassay of Krey et al. (1975) with a highly specific B antiserum (B3-163, Endocrine Sciences, Tarzana, CA), [3H-B] (101 Ci/mmol; New England Nuclear, Boston, MA) as tracer and 10 ml of plasma. The minimum level of detection of the assay was 10 pg/ml. The antiserum cross-reacts slightly with deoxycorticosterone (4%), but not with cortisol (<1%). Separation of bound from unbound hormone was achieved using dextran-coated charcoal. Samples were then decanted into mini-scintillation vials, filled with 4.5 ml of Liquiscint (National Diagnostics, Sommerville, NJ) and radioactivity determined in a Packard scintillation counter at 56%

efficiency. The intra- and inter-assay coefficients of variation were 3.2% and 3.9% respectively.

Plasma ACTH was measured by a modified radioimmunoassay described by Walker et al. (1990) with an ACTH antiserum (IgG Corp., Nashville, TN) and [^{125}I]-ACTH (Incstar, Stillwater, MN) as tracer. The ACTH antibody cross-reacts 100% with $ACTH_{1-39}$, $ACTH_{1-18}$ and $ACTH_{1-24}$, but not with $ACTH_{1-16}$, β–endorphin, α- and ß-MSH and α- and ß-lipotropin (<1%). Plasma(25 µl) was incubated for 48 h at 40C with antiserum and tracer, after which precipitation serum (Peninsula Laboratories, Belmont, California) was added and incubated overnight. Bound peptide was obtained by centrifugation at 5000 x g for 45 minutes. The minimum level of detection of the assay was 10 pg/ml; inter-and intra-assay variabilities were 4.0% and 2.8% respectively.

Plasma GH levels were determined in duplicate by double antibody RIA using materials supplied by the NIDDK (Bethesda, MD). The averaged plasma GH values are reported in terms of the rat GH reference preparation (rGH RP-2). The standard curve was linear between 0.62-320 ng/ml. The intra- and inter-assay coefficients of variation were 8.1% and 8.7%, respectively, for duplicate samples of pooled plasma containing a mean GH concentration of 11.5 ng/ml.

Statistical analyses

The results were analyzed by repeated measures and factorial analyses of variance (ANOVA), and paired and unpaired Student's t-tests. Scheffé post-hoc tests were performed when appropriate. Integrated hormone levels were determined with the trapezoidal rule and the data expressed over time of sampling. $P<0.05$ was considered significant.

Results

Effects of High-Fat Diet on Spontaneous Plasma Corticosterone (B) and Growth Hormone (GH) Profiles

Plasma B levels measured every 15 minutes over 6 hours and averaged over 2-h blocks (10:00-12:00 h; 12:00-14:00 h and 14:00-16:00 h) are shown in Figure 39. All rats displayed the typical circadian elevation in basal plasma B with increasing levels observed during the later hours of the day; spontaneous basal plasma B values obtained in the 10:00-12:00 h time period were significantly ($p<0.05$) lower in all groups when compared to their respective profiles from either from 12:00-14:00 h or 14:00-16:00 h period. However, when compared to normal-fed controls, mean 2-hour plasma B levels were markedly elevated in both groups of high-fat fed rats (Fig. 39). Rats fed a high-fat diet for 1 week showed 2- to 3-fold increases in plasma B levels throughout the sampling period. After 3 weeks exposure to high-fat, significant elevations in B were observed in the 12:00-14:00 h and 14:00-16:00 h sampling periods when compared to those of normal-fed controls . Interestingly, mean plasma B profiles obtained from rats fed the high-fat diet for 7 days were significantly ($p<0.05$) higher at both the 10:00-12:00 h and 12:00-14:00 h phases than the profiles obtained from rats fed the high-fat diet for 21 days.

In contrast to the effects observed on plasma B, exposure to a high-fat diet for either 7 or 21 days failed to significantly alter spontaneous GH profiles. As shown in Table 8, there were no significant differences in either GH peak amplitude, GH trough value or mean 6-h plasma GH levels between the three groups. Daily body weight gain and food intake were also similar across the three groups (Table 8). These latter data essentially reflect what was found in all other studies (stress response and negative feedback).

Effects of High-Fat Diet on Plasma ACTH and B Responses to Restraint

Plasma ACTH and B responses immediately before, during and up to two hours following the termination of restraint are shown in Fig 40. Compared to animals fed the control diet, rats fed the high-fat diet for one week showed elevated levels of ACTH throughout the sampling period which reached significance at 60 minutes following termination of stress ($p<0.05$) (see Fig. 40A). Overall, the integrated levels of plasma ACTH were significantly ($p<0.05$) increased in animals fed the high-fat diet for one week as compared to controls (Fig. 41A). One-week high-fat fed rats also showed a significant elevation in plasma B at 60 minutes following the termination of restraint (Fig. 40B) in addition to significantly ($p<0.05$) higher integrated levels of B compared to controls (Fig. 41B).

After nine weeks on the high-fat diet, a significant ($p<0.05$) elevation in plasma ACTH was observed at 20 and 120 minutes after the termination of stress (Fig. 42A) and integrated ACTH levels were significantly ($p<0.05$) elevated when compared to controls (Fig. 43A) . High-fat fed rats also showed significant ($p<0.05$) elevations in plasma B at 60 and 120 minutes post-stress, as shown in Fig. 42B, as well as significantly ($p<0.05$) higher integrated B levels as compared to control-fed animals (Fig.43B).

Finally, rats fed for 12 weeks on the high-fat diet did not exhibit a significant increase in plasma ACTH compared to control rats (Fig. 44A; integrated levels shown in fig. 45A) but did show significant ($p<0.05$) elevations both in plasma B at 60 and 120 minutes post termination of stress (Fig. 44B) and integrated levels of B (Fig. 45B). Plasma B levels in high-fat fed rats were also elevated 20 minutes following the termination of restraint, although this difference failed to reach significance ($0.1<p>0.05$).

Basal and Stress-Induced Plasma Fatty Acid (FA) and Blood Glucose Concentrations

Figure 46A illustrates basal FA levels in 1, 9, 12 week high-fat and control fed animals. Basal FA levels were significantly ($p<0.03$) elevated in 1 and 9 week high fat-fed animals as compared to controls but no significant differences were found at 12 weeks. Plasma FA responses prior to, and following exposure to a 20 min period of restraint, are shown in Fig. 46B. High-fat fed animals maintained on the diet for 1 week showed significant ($p<0.03$) elevations in FA immediately prior to (-20) and at 5 and 10 minutes after the onset of restraint as compared to controls. Upon termination of restraint, rats fed the high-fat diet maintained significantly ($p<0.03$) elevated FA levels as compared to controls. Integrated FA levels were augmented two-fold ($p<0.05$) in fat-fed rats (1110 ± 112 μEq/L/min) vs controls (585 ± 50 μEq/L/min).

Basal plasma glucose levels obtained at the time of sacrifice were similar in fat-fed versus control-fed rats at all time points (Fig. 47A). While high-fat fed rats showed a significant ($p<0.05$) elevation in plasma glucose 5 minutes into the restraint (Fig. 47B), there were no significant differences in integrated plasma glucose levels when compared to controls (110 ± 5 mg/dl/min for high-fat fed rats and 103 ± 4 mg/dl/min for controls).

Similar FA and glucose responses to stress were found in rats fed for 9 weeks with the high-fat diet (data not shown); integrated FA levels were 1170 ± 82 μEq/L/min and 871 ± 66 μEq/L/min in rats fed the high fat and control diets, respectively ($p<0.05$) whereas the integrated level of plasma glucose in high-fat fed rats was 103 ± 3 mg/dl/min as compared to 92 ± 3 mg/dl/min in controls.

Rats exposed to the high-fat diet for 12 weeks exhibited no significant differences in stress-induced FA levels when compared to controls (integrated FA levels: 1091 ± 131 μEq/L/min vs. 947 μEq/L/min). Integrated plasma glucose levels were also similar in the two groups: 101 ± 3 mg/dl/min vs. 107 ± 2 mg/dl/min for controls (data not shown).

Insulin Sensitivity Test

Figure 48 shows plasma glucose levels prior to ("pre") and following an injection of 0.125 Units/kg of insulin in rats exposed to the high-fat or control diets for 12 weeks. No significant differences in plasma glucose concentrations were found prior to insulin administration. Control-fed rats demonstrated a significant decrease in blood glucose in response to insulin administration. In contrast, fat-fed rats failed to respond to insulin at 10:15 h and this resulted in plasma glucose levels being significantly ($p<0.02$) higher at 15 and 30 minutes after injection in high-fat fed animals, compared with controls. High-fat-fed animals showed significantly ($p<0.05$) elevated integrated plasma glucose levels (95 $\pm$4 mg/dl/min) as compared to controls (75 $\pm$ 6 mg/dl/min).

Glucocorticoid Receptor Binding Densities

Examination of glucocorticoid receptor (GR) binding in brain regions known to be implicated in glucocorticoid negative feedback regulation of HPA activity revealed no significant differences in GR binding densities in either hippocampus, frontal cortex or pituitary after one week on the diet although GR binding in the hypothalamus was significantly lower ($p<0.05$) in high-fat fed animals on the diet as compared to controls (Fig. 49A). After 9 (Fig. 49B) and 12 (Fig. 49C) weeks exposure to the high-fat or control diets GR binding densities were similar in all brain areas studied.

Feedback of Exogenous B

Table 9 shows both basal plasma ACTH and B concentrations, as well as stress-induced ACTH levels, in rats that were ADX and replaced with a 0%, 25% or 50% B pellets, and exposed to both diets. Replacement with B-pellets effectively decreased plasma ACTH levels following ADX. However, there were no significant differences in either basal ACTH or B levels, or stress-induced ACTH, between ADX rats fed the high-fat or control-fed animals.

Table 7. Composition of 20% fat (high-fat) and 4% fat (control) diets.

Ingredient	Control Diet (g/100g)	High-Fat (g/100g)
Brewer's Yeast	1.0	1.0
Fish Meal	18.0	18.0
Oats (rolled)	15.0	15.0
Bran (ground)	12.5	12.5
Wheat germ	16.0	16.0
Starch Corn	24.5	8.5
Corn Oil	4.0	20.0
Skim Milk Powder	4.0	4.0
Custom Vitamin Mix	0.01	0.01
Custom Mineral Mix	4.9	4.9

Control diet=4.0 Kcal/gram *Fat Diet=4.8 Kcal/gram*

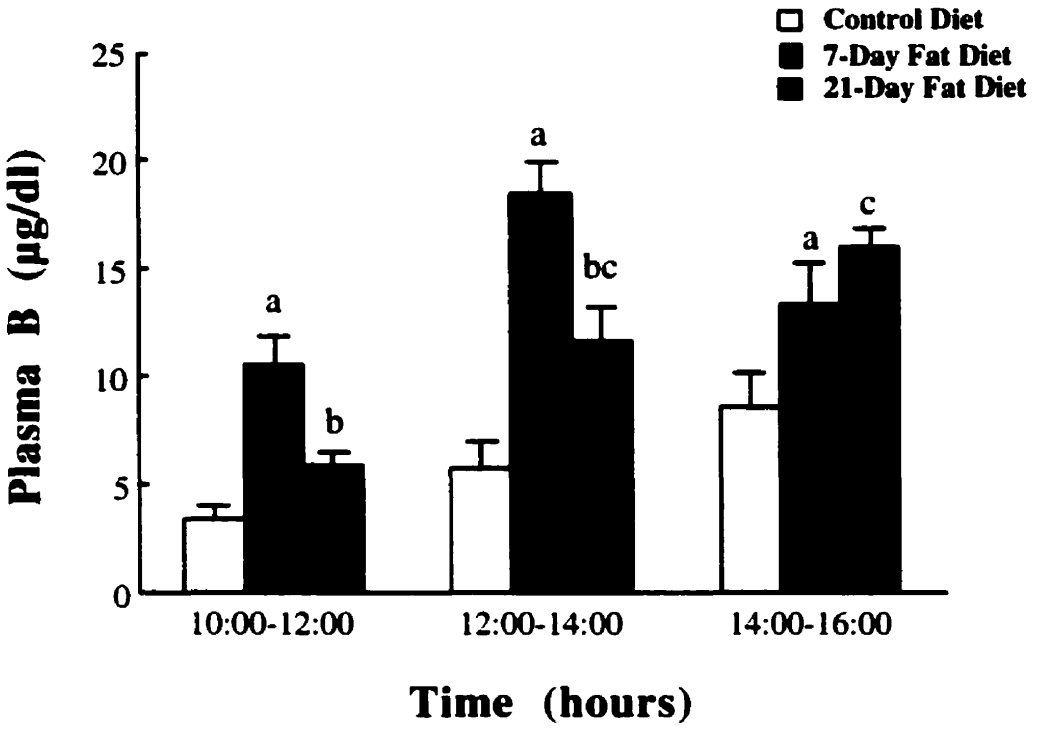

Figure 39 Two-hour plasma corticosterone (B) profiles (mean ± SEM) in freely-moving rats exposed to high-fat diet for 7 (n=8) or 21 (n=6) days vs. controls (n=6) across the day. a 7 day fat-fed rats significantly different from controls at; b 21 day fat-fed vs. 7 day fat-fed different; c 21-day fat-fed rats differ from controls .

Table 8. Effects of High-Fat Diet on Mean (± SEM) GH Secretory Dynamics, Body Weight Gain and Food Intake.

Experimental Group	n	GH Peak Amplitude (ng/ml)	GH Trough Level (ng/ml)	Mean 6-h Plasma GH Level (ng/ml)	Daily Body Weight Gain (g)	Daily Food Intake (g)
Control Diet-7 to 21 days	6	180.5 ± 31.0	1.3 ± 0.1	39.0 ± 4.9	6.1 ± 1	23.9 ± 1.2
High-Fat Diet-7 days	10	165.5 ± 28.3	1.4 ± 0.2	42.6 ± 8.0	5.8 ± 0.8	24.7 ± 0.7
High-Fat Diet-21 Days	6	147.1 ± 42.3	1.3 ± 0.1	34.3 ± 9.0	5.5 ± 0.3	20.3 ± 1.4

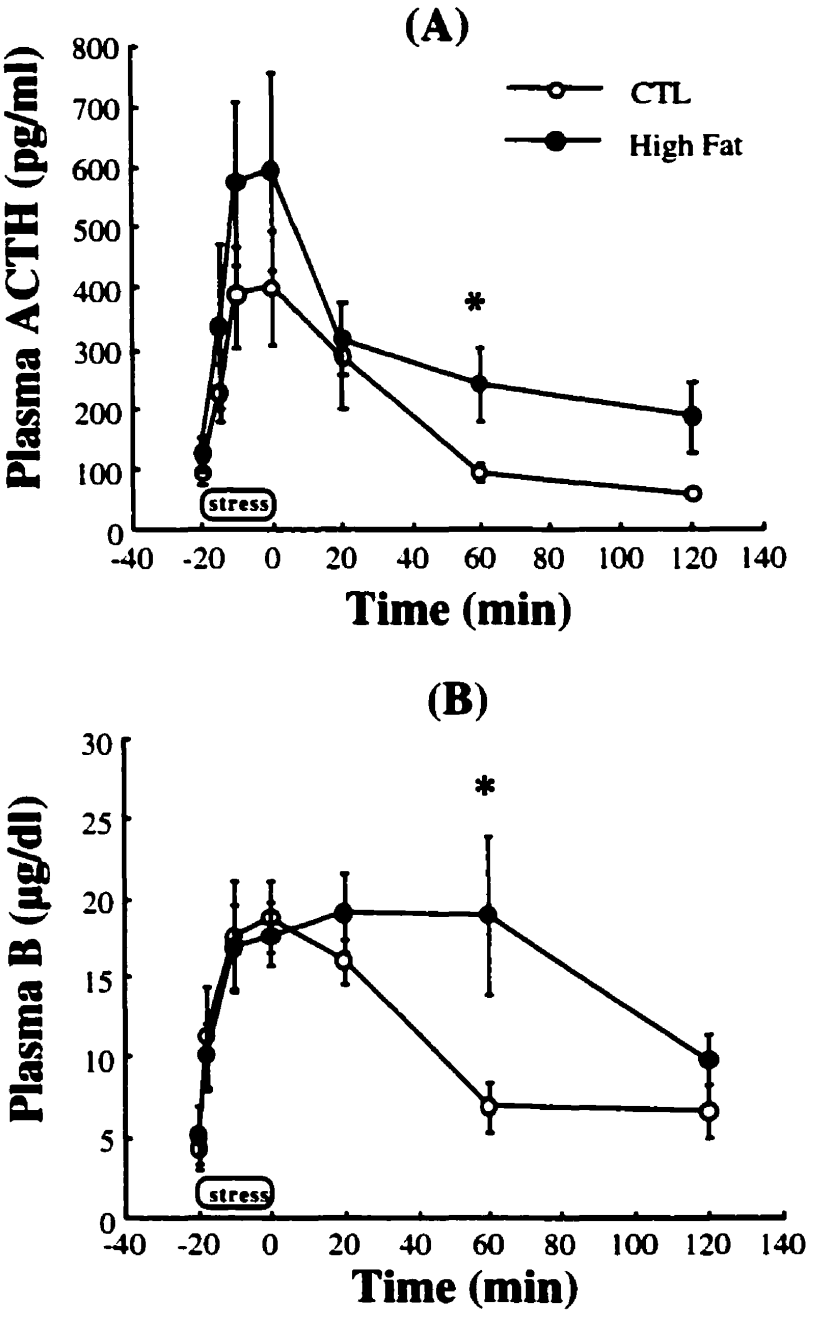

Fig. 40 Mean + SEM of plasma ACTH (panel A) and corticosterone (B) (panel B) responses prior to, during and at various times following, a 20-minute period of restraint. Rats fed either a high-fat diet (n=9 in each group) or control diet (n=9 in each group) for 1 week are represented. Stress box indicates 20 minute period of restraint. * indicates points that differ significantly at $p<0.05$.

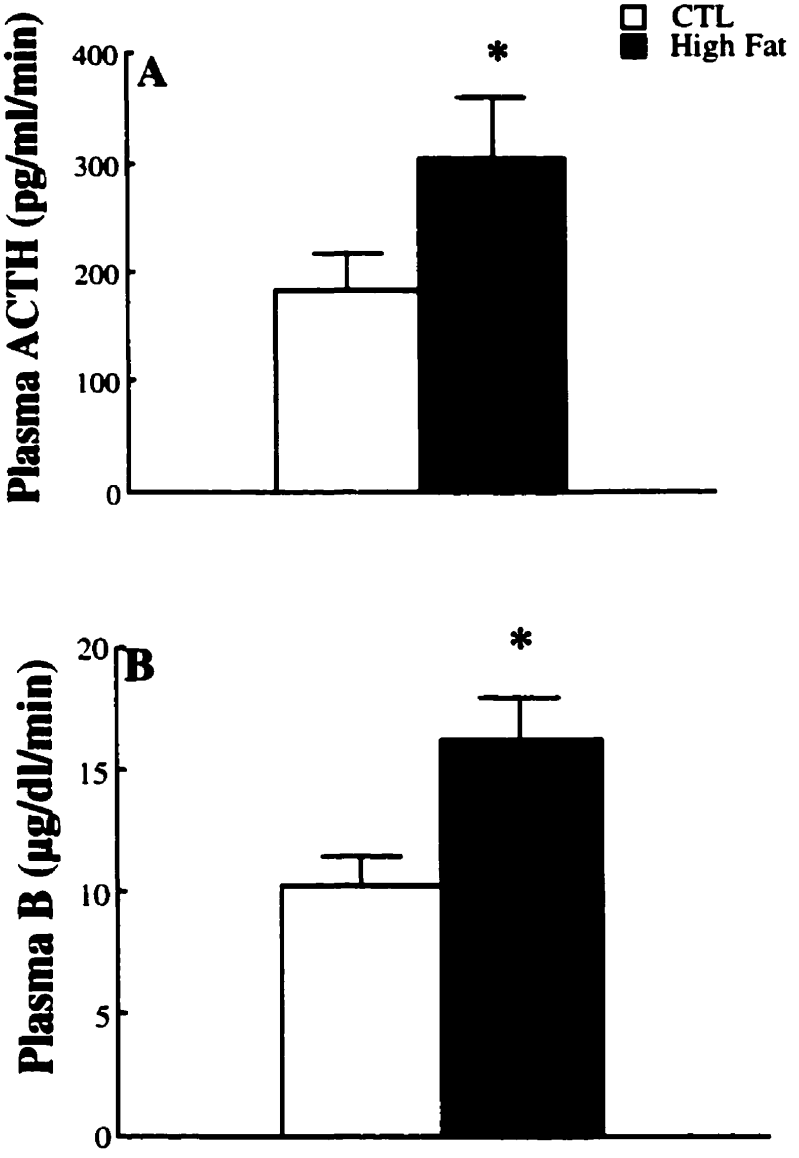

Fig. 41 Mean + SEM of integrated (area under the curve) plasma ACTH (Panel A) and corticosterone (B) (Panel B) responses to a 20-minute period of restraint. Rats fed a high-fat diet (n=9 in each group) or control diet (n=10) for 1 week are represented. * indicates points that differ significantly at $p<0.05$.

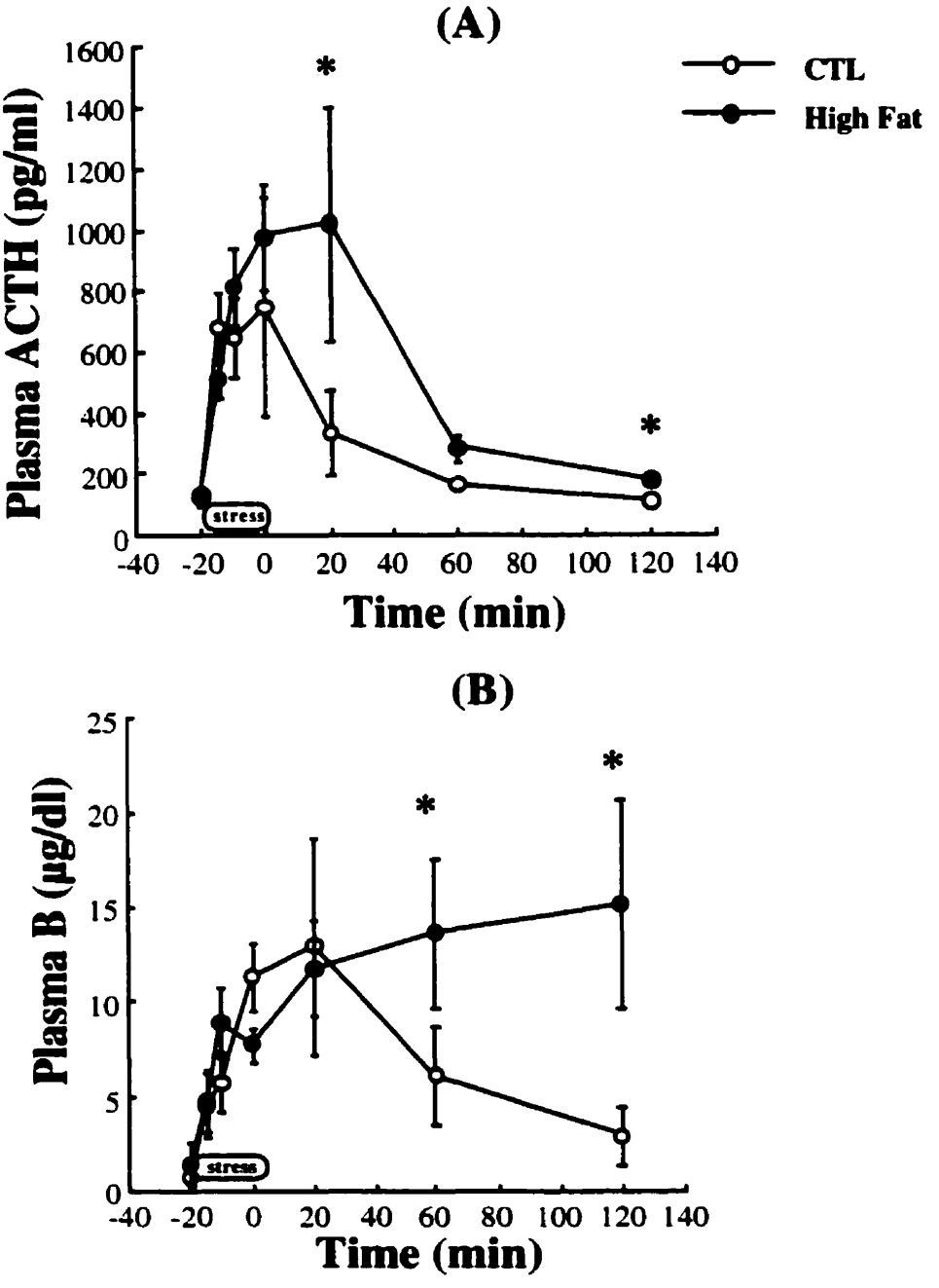

Fig.42. Mean + SEM of plasma ACTH (panel A) and corticosterone (B) (panel B) responses prior to, during and at various times following, a 20-minute period of restraint. Rats fed either a high-fat diet (n=9 in each group) or control diet (n=9 in each group) for nine weeks are represented. Stress box indicates 20 minute period of restraint. * indicates points that differ significantly at $p<0.05$

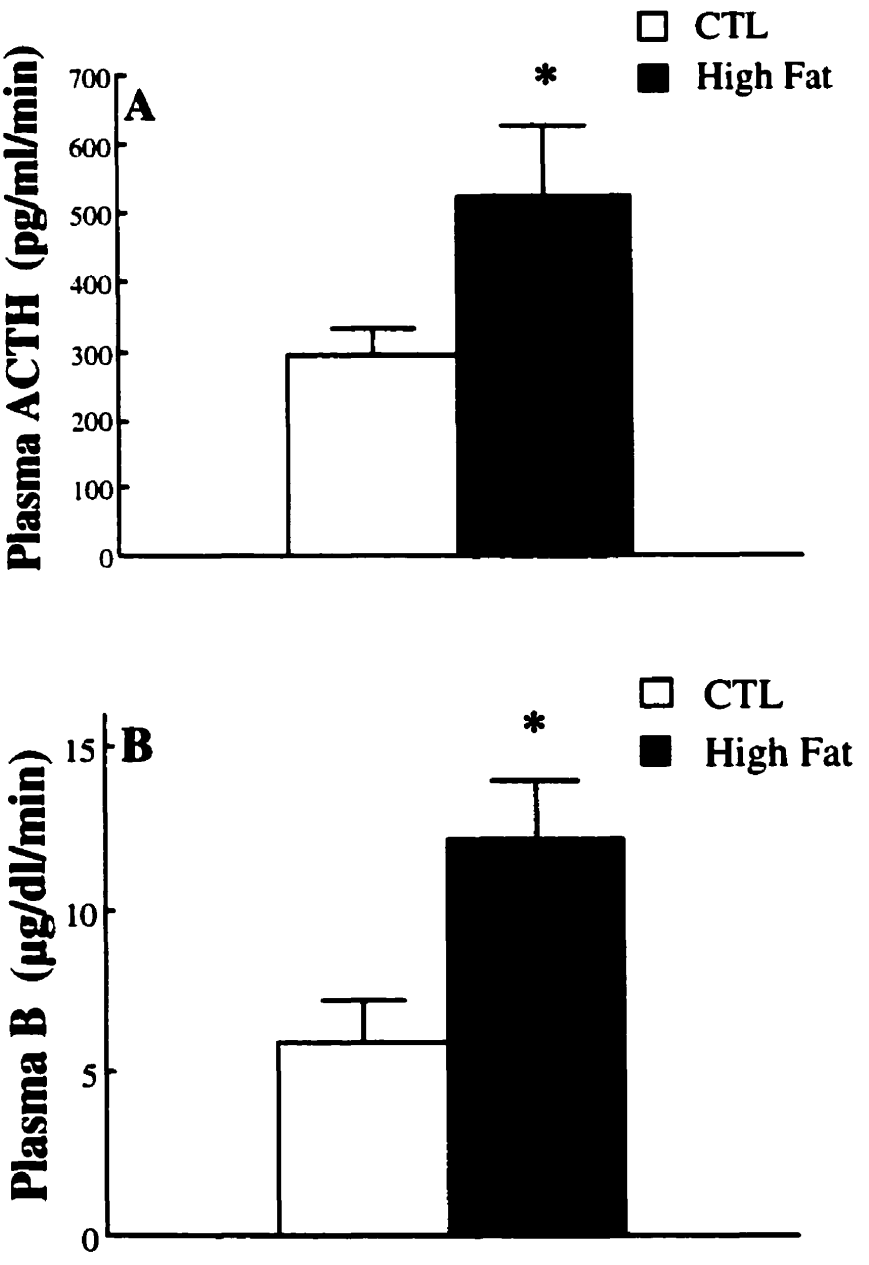

Fig. 43 Mean + SEM of integrated (area under the curve) plasma ACTH (panel A) and corticosterone (B) (panel B) responses to a 20-minute period of restraint. Rats fed a high-fat diet (n=9 in each group) or control diet (n=10) for nine weeks are represented. * indicates points that differ significantly at $p<0.05$.

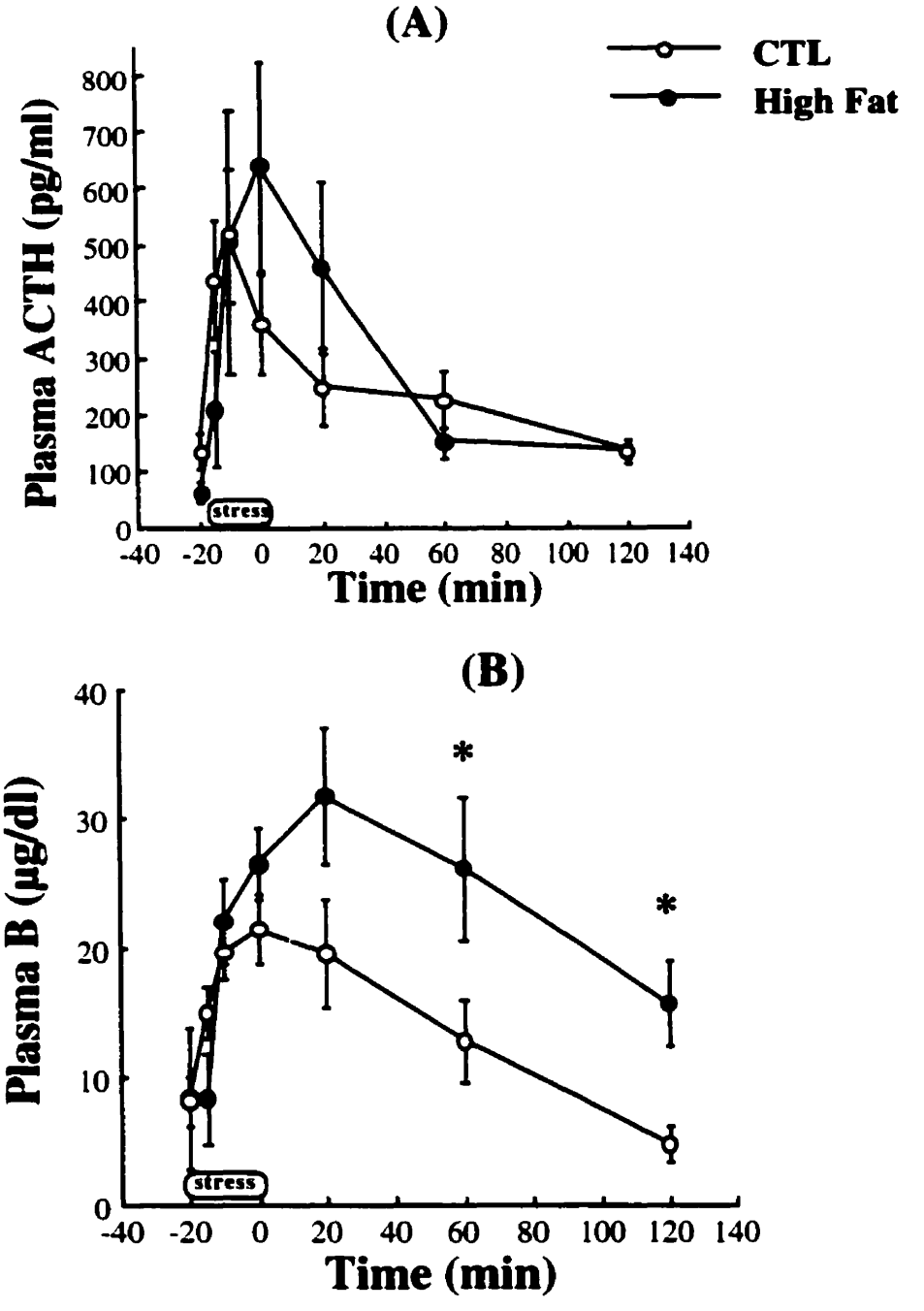

Fig. 44 Mean + SEM of plasma ACTH (panel A) and corticosterone (B) (panel B) responses prior to, during and at various times following, a 20-minute period of restraint. Rats fed either a high-fat diet (n=9 in each group) or control diet (n=9 in each group) for twelve weeks are represented. Stress box indicates 20 minute period of restraint. * indicates points that differ significantly at $p<0.05$. Note in panel B, that at 20 min. following the termination of stress, $0.1<p>0.05$.

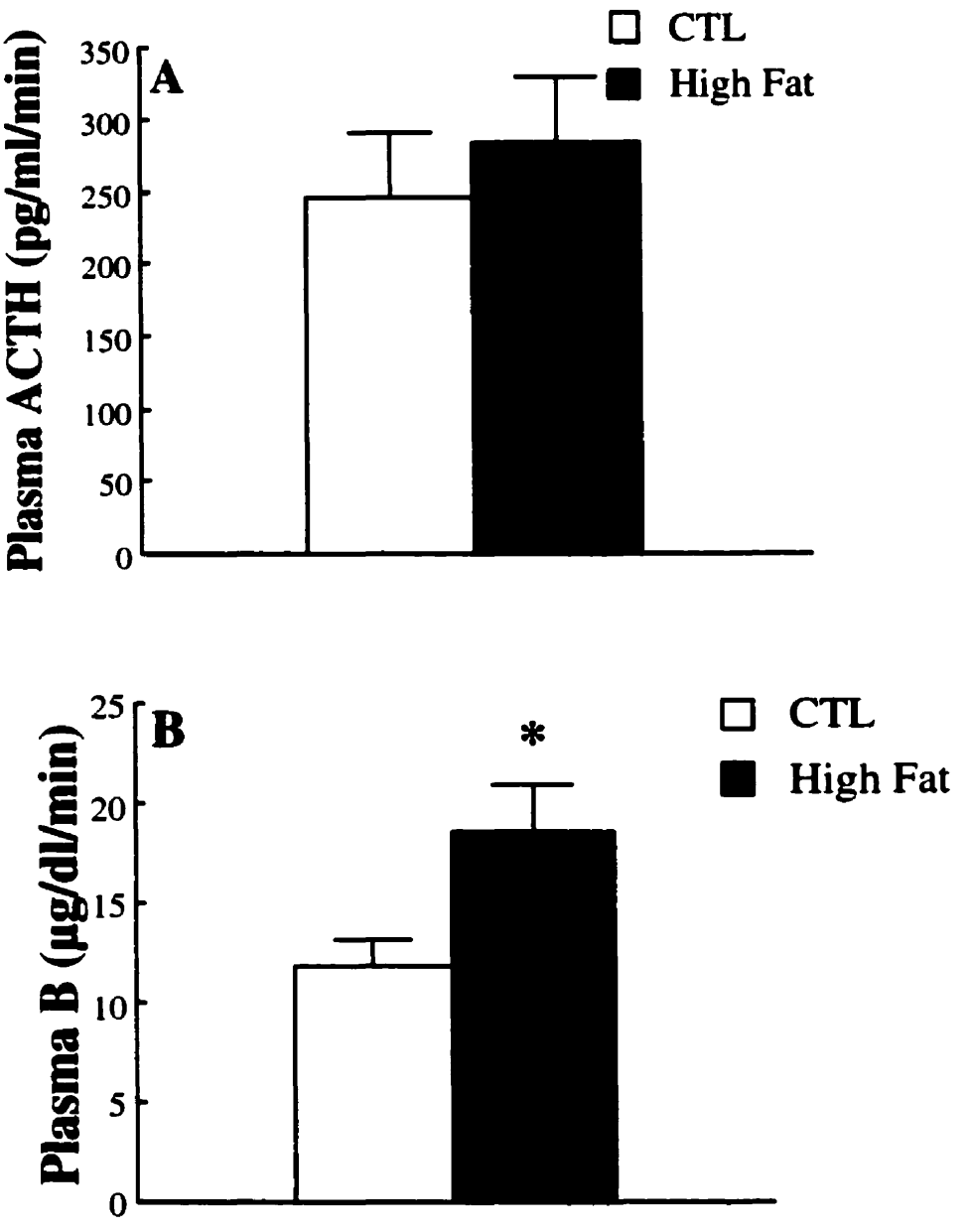

Fig. 45 Mean + SEM of integrated (area under the curve) plasma ACTH (panel A) and corticosterone (B) (panel B) responses to a 20-minute period of restraint. Rats fed a high-fat diet (n=9 in each group) or control diet (n=10) for twelve weeks are represented. * indicates points that differ significantly at $p<0.05$.

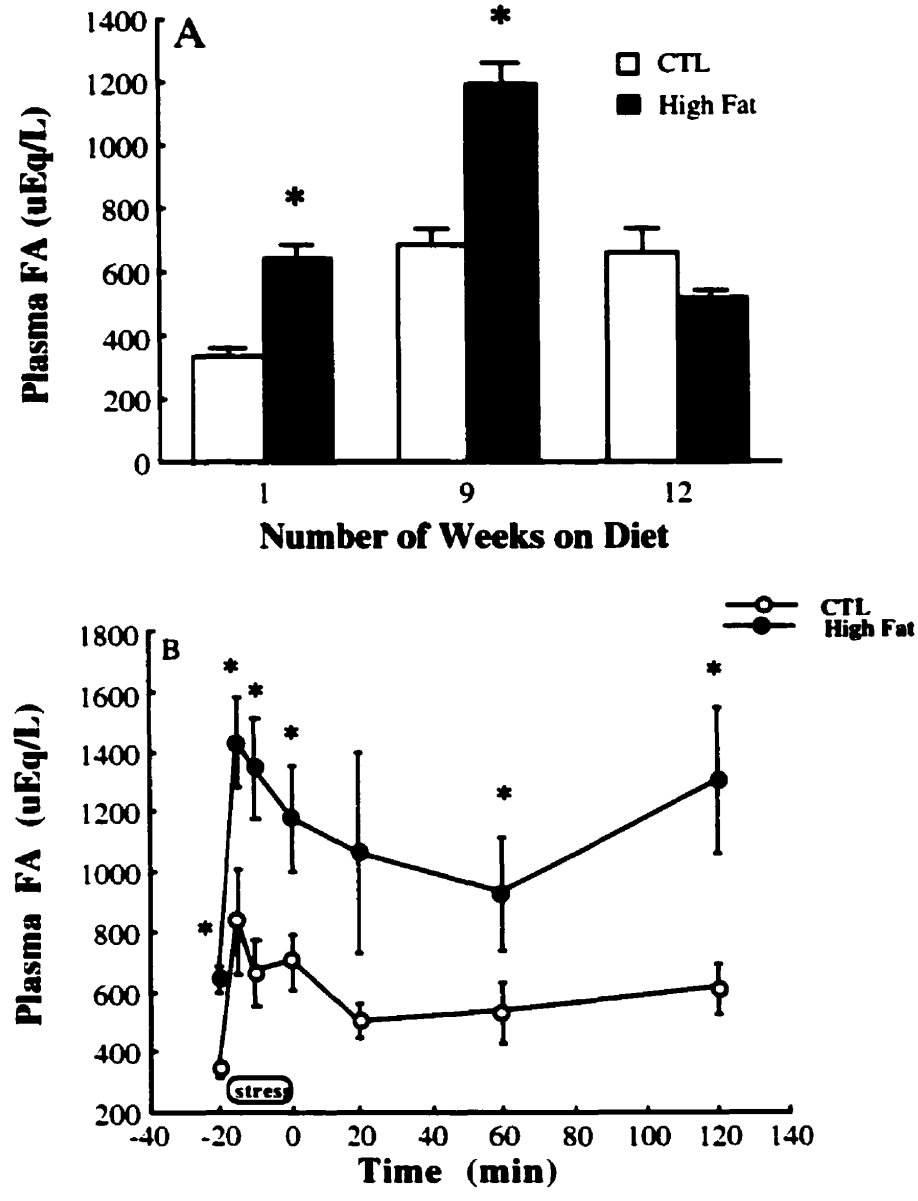

Fig. 46 Mean + SEM basal (A) and stress-induced (B) plasma fatty acid (FA) levels in rats fed either the high-fat (n=10) or control (n=10) diets. Top panel represents basal FA levels obtained from trunk blood at time of sacrifice. Bottom panel represents FA response to a 20-minute period of restraint (stress box) in animals fed the high-fat (n=9) or control (n=10) diets for one week. * indicates points that differ significantly at $p<0.03$.

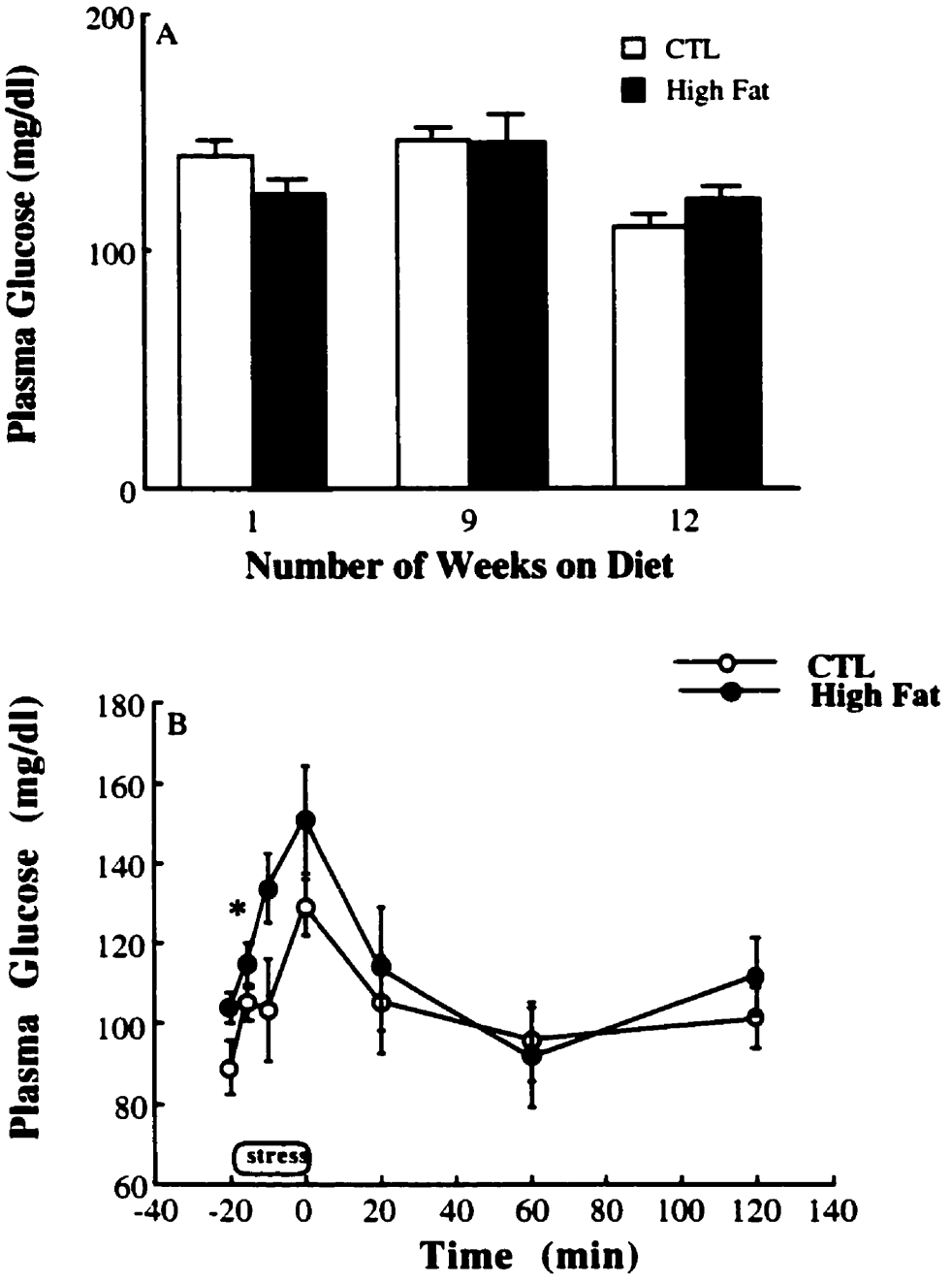

Fig. 47 Mean + SEM basal (A) and stress-induced (B) plasma glucose concentrations in rats fed either the high-fat (n=10) or control (n=10) diets. Top panel represents basal glucose levels obtained from trunk blood at time of sacrifice. Bottom panel represents the plasma glucose response to a 20-minute period of restraint (stress box) in animals fed the high-fat (n=9) or control (n=10) diets for one week. * indicates points that differ significantly at $p<0.05$.

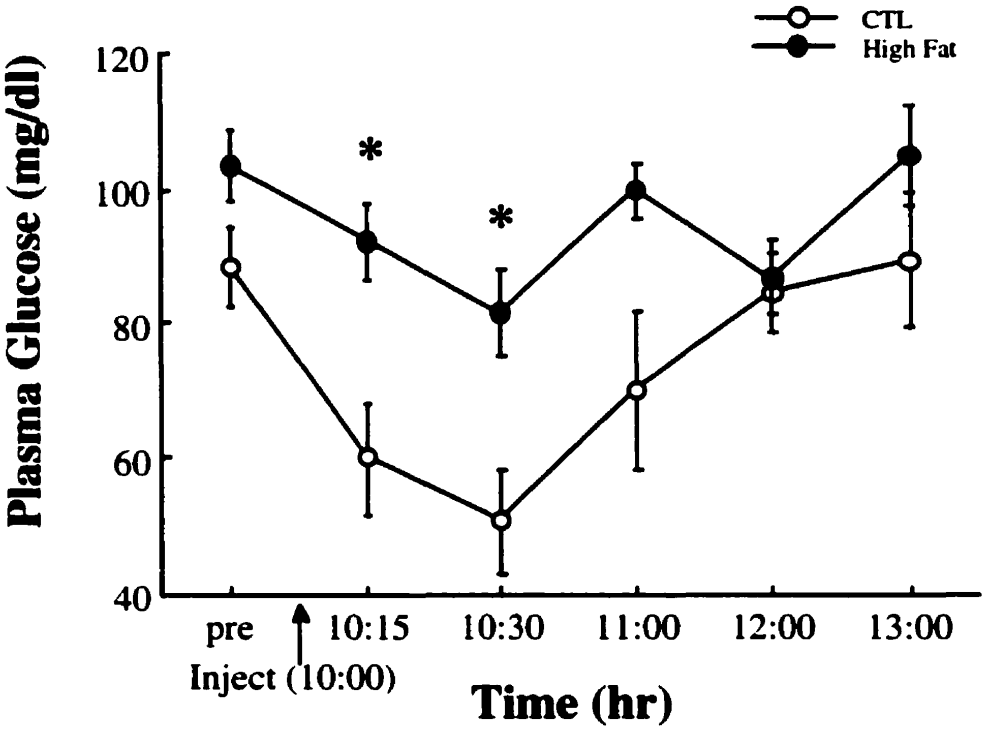

Fig. 48 Mean + SEM blood glucose levels prior to and up to 3 hours following a 0.125 U/kg i.p. injection (arrow indicates time of injection) of insulin in control (n=8) and fat-fed (n=9) rats * indicates points that differ significantly at $p<0.02$.

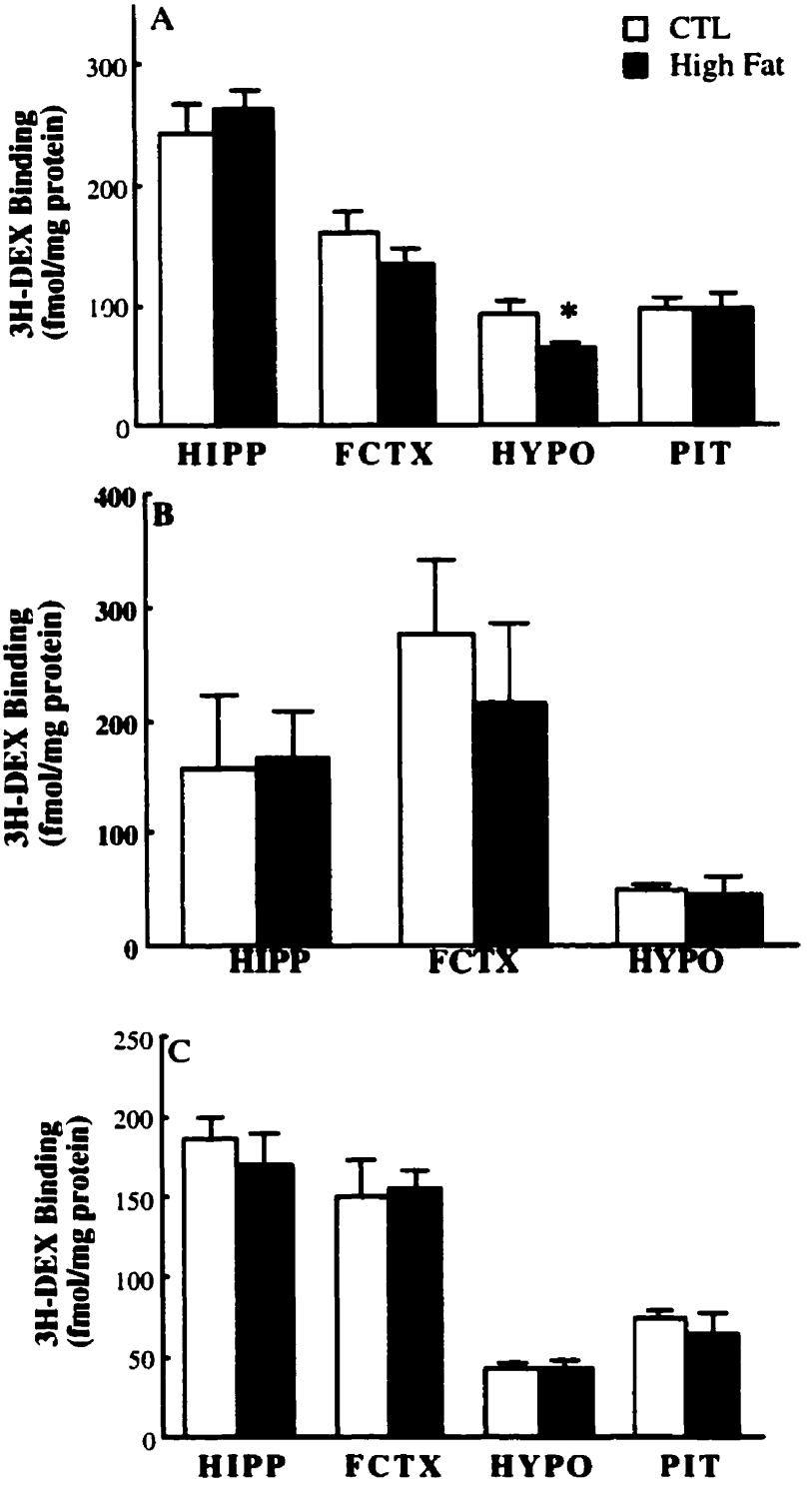

Fig. 49. Mean + SEM glucocorticoid receptor binding capacity (DEX binding) in various brain regions from rats fed either the high fat or control diets for one (A), nine (B) or 12 (C) weeks (n=8 in each group). * indicates points that differ significantly at $p<0.05$. HIPP=hippocampus, FCTX=Frontal Cortex, HYPO=Hypothalamus, PIT=Pituitary.

Table 9 . Effects of High-Fat Diet on Mean (± SEM)Basal and Stress-Induced Plasma ACTH and B Levels in B Pellet-Replaced Rats

Experimental Group	n	O minute ACTH (pg/ml)	30 Minute ACTH (pg/ml)	Basal B (ug/dl)
Control Diet (ADX+0%)	6	490 ± 107	1711 ± 152	0.2 ± 0.1
Control (ADX+25%)	6	152 ± 73	1312 ± 110	2.5 ± 0.5
Control (ADX + 50%)	6	72 ± 21	154 ± 15	5.0 ± 1.5
High-Fat (ADX + 0%)	7	550 ± 97	2100 ± 154	0.2 ± 0.1
High-Fat (ADX+25%)	7	110 ± 47	1100 ± 55	2.0 ± 1.0
High-Fat (ADX+ 50%)	7	75 ± 21	201 ± 27	6.9 ± 1.5

Discussion

These results demonstrate that both basal and stress-induced HPA activity were altered by both short- and long-term exposure to a high-fat diet. Free-moving, high-fat fed animals maintained on the diet for one and three weeks and sampled throughout the day showed 2-3 fold increases in plasma B concentrations between 10:00 and 16:00 h when compared to controls. Rats fed the high fat diet for one, nine and twelve weeks also showed significant elevations in plasma ACTH, B and fatty acid levels at 20, 60 and 120 min following the termination of restraint and significant augmentation in the overall integrated ACTH, B and FA responses to restraint stress, when compared to normal-fed controls. In contrast, there were no significant alterations in any parameters of the spontaneous GH secretory profiles after 1 and 3 weeks on the high-fat diet, providing support for the specificity of the effects on the HPA axis.

Interestingly, the pattern of effects of the high-fat diet is similar to that observed following chronic stress. Basal HPA activity is increased in rat models of chronic stress, such as continuous cold exposure or streptozotocin-induced diabetes, and this effect is most apparent in the light phase of the cycle, i.e. the nadir in HPA activity (Scribner et al., 1991). Moreover, chronic stress facilitates HPA responses to subsequent acute stressors (Akana et al., 1992). High fat diets clearly augmented both ACTH and B responses to acute stress. The effects of chronic stress (enhanced ACTH and B responses to stress) are not associated with alterations in delayed glucocorticoid negative-feedback sensitivity. Accordingly, high-fat fed rats of the present study also did not differ from controls in delayed feedback sensitivity. Thus in terms of HPA activity, sustained periods of increased fat consumption appear to function as a chronic stressor. The lack of effect of increased fat consumption on GH secretory profiles is consistent with earlier reports demonstrating that plasma GH concentrations are not altered in conditions of chronic stress (Hermanussen et al., 1995; Lechin et al., 1994).

As in other chronic stress models, there appeared to be a small amount of adaptation to high-fat feeding. After 12 weeks exposure to high-fat, animals failed to show a significantly elevated ACTH response to stress, despite a significantly augmented B response to the restraint. Animals fed the high-fat diet for 12 weeks also did not differ in basal FA levels when compared to controls. However, while it is possible that these particular responses adapted following longer exposures to fat feeding, adrenal GC responses remained elevated. Moreover, these animals were clearly non-responsive to insulin challenge; rats tested after 12 weeks exposure to high-fat demonstrated a significant resistance to insulin as evidenced by a lower decline in blood glucose levels following the administration of insulin. This finding supports the severity of the effects of long-term (12 weeks) fat feeding on insulin and glucose dynamics and underscores the functional significance of this high fat model in rodents. While high-fat diet-induced insulin insensitivity has been shown in models where exposure to the diet has been more prolonged, our results suggest that relatively short exposures to high-fat diets can induce severe changes in insulin dynamics without any major change in circulating basal blood glucose levels, a phenomenon often seen in the clinical setting (Reaven, 1988).

High-fat diets have been reported to increase serum FA concentrations which, in turn, may act to antagonize the action of insulin (Ruderman et al., 1969). Feeding experimental animals with high-fat diets induces insulin resistance and impairs intracellular glucose metabolism by a variety of mechanisms. The binding of insulin to its receptor initiates glucose transport into fat and muscle cells. Insulin stimulates glycogen synthesis, glycolysis and glucose oxidation, while high-fat diets impair all of the intracellular routes of glucose disposal (Ernest et al., 1962; Hissin et al., 1982). High FA may act directly to reduce the number of insulin receptors in certain tissues (Anderson and Bridges, 1984). High-fat diets also decrease the activities of the key enzymes involved in glycolysis (Anderson and Herman, 1975) while, at the same time, stimulating gluconeogenesis (Ruderman et al., 1969). The present findings show that high-fat fed rats exhibit

elevations in both basal and stress-induced FA. FA levels were elevated prior to, during and immediately following the termination of restraint in the high-fat fed animals; this elevation in FAs may mediate changes in both insulin sensitivity and HPA function. The high FA levels appeared to coincide with higher basal and stress-induced ACTH. Since ACTH has been shown to be an stimulus for lipolysis, the ACTH response to stress may best predict the FA levels.

Widmaier et al. (1992) showed that elevations in FA, achieved by infusions of intralipid, raise plasma levels of ACTH and B. Fatty acids have direct electrophysiological effects on cells of the central nervous system (CNS) and are taken up by cells in the brain (Love et al., 1985; Oomura, 1976). Oomura (1976) demonstrated that electroapplication of FA into the ventromedial hypothalamus (VMH) inhibited neuronal firing rates in that area, and Dallman (1984) has shown that the cells of the VMH exert an inhibitory control on the HPA. Thus FA may act to alter hypothalamic regulation of the HPA axis. We propose that high-fat induced elevations in FA may be partly responsible for the elevation in basal B as well as the increased stress-induced hypersecretion of both B and ACTH. Further, because GCs tend to stimulate lipolysis, elevations in B may further stimulate the production of FA, which are already elevated due to the fat content of the diet. This may render the animal even more resistant to insulin, since elevations in FA tend to reduce the number of insulin receptors on various tissues, thereby increasing the dependence on GCs for homeostasis and stimulating gluconeogenesis. Taken together, these findings suggest a feed-forward cascade involving FA, GC and insulin dynamics.

Exposure to high-fat diets did not alter B negative feedback sensitivity. We did find differences in hypothalamic GR levels following 1 week, but not 9 or 12 weeks, of high-fat feeding. The absence of differences at 9 and 12 weeks of feeding might be attributable to the poor resolution of our technique used for the binding assays. We cannot, therefore, preclude the possibility of differences in other forms of feedback, such as fast feedback. Fast feedback represents a process whereby rapidly increasing levels of B dampen ACTH

release (Keller-Wood and Dallman, 1984). Fatty acids may play a role here. The binding of dexamethasone (DEX) to the rat cytosolic GR can be modified by FA, for ex. FA inhibit the binding of DEX to GR in the liver as a function of increasing dose (Vallette et al., 1991). Since GCs stimulate lipolysis FA could exert a feedback control on GC by modulating binding of the hormone to the receptor. Also, recent work shows that the binding of GC in vivo to GR is reduced in immature rats after plasma FA is increased by stimulating lipase activity (Haourigiu et al., 1994). The elevations in FA levels occur under more dynamic conditions of stress, and these conditions would be expected to alter fast, but probably not delayed, forms of feedback. While this idea clearly remains to be tested, it does provide another potential mechanism whereby high fat diets could modulate HPA responses to stress.

Glucocorticoids can antagonize the effects of insulin, produce insulin insensitivity and decrease glucose uptake in tissues. However the combination of elevated GCs with concurrent increases in insulin further enhances energy deposition through FA and glycogen synthesis and the activity of lipoprotein lipase in adipose tissue (Brindley and Rolland, 1989). An increased GC control of metabolism is characteristic of many of the risk factors for premature atherosclerosis. The GC-insulin antagonism stimulates the secretion of vLDL and decreases hepatic uptake of LDL (Wang et al., 1995). High-fat fed rats are insulin resistant and therefore may be at greater risk for the development of Syndrome X and non-insulin dependent diabetes mellitus (NIDDM).

A strong correlation exists between consumption of a diet high in fat and many cancers such as breast, colorectal, pancreatic, prostatic and uterine (Schapira, 1991). Munck et al. (1984) demonstrated that GCs inhibit production of interferon which augments natural killer (NK) cell activity and activates macrophages for clearance of bacterial pathogens and anti-body tagged host cells. Thus, in addition to obvious implications for heart disease, increased glucocorticoid production may mediate some of the effects of high fat diets on tumor development in addition to the onset and progression of

related pathologies. If one considers that high-fat feeding increases GC and FAs and decreases insulin sensitivity in an atherosclerosis-prone animal (eg. a human), then these stress-induced changes should aggravate the atherosclerosis.

In summary, the results of the present study demonstrate that high-fat feeding augments both the ACTH and B responses to acute stress, as well as increases in basal B secretion, without any significant alterations in B negative feedback efficacy. Further, we have shown that high-fat feeding results in insulin resistance and elevations in both basal and stress-induced FA and blood glucose concentrations. Taken together, these findings provide initial support for the view that enhanced exposure to counter-regulatory hormones can mediate the effects of high-fat diets. These findings may be of considerable clinical importance since stressful events not only stimulate HPA activity, but may also increase fat consumption, leading to a potentially dangerous metabolic cascade. Long-term high-fat consumption, through augmented GC production, may then accelerate the aging process by rendering both central and peripheral regulatory systems vulnerable to the effects of the catabolic effects of increased B.

Study 4 The Effects of Prolonged High-Fat Feeding on HPA and Metabolic Function in the Aged Rat

High fat diets are associated with a Syndrome X-like state that includes hypertriglyceridemia, increased LDL and vLDL, decreased high density lipoproteins (HDL), abnormal glucose production, hyperinsulinemia and insulin resistance (Reaven, 1988). This constellation of symptoms is often seen in middle-aged or elderly individuals and it emerges as a result of life-long dietary and lifestyle factors. In fact, numerous studies in rats, monkeys and humans suggest that it is not aging per se but a high content of fat in the diet, body mass index and a sedentary lifestyle that contribute substantially to the development of many of these perturbations. (Zavaroni et al, 1986; Barnard et al., 1995).

In addition to their effects on blood lipids, glucose and insulin, high fat diets also influence HPA activity, elevating adrenal glucocorticoid (GC) production (see previous study; Brindley et al., 1981; Hulssman, 1978; Pascoe et al., 1990; Tannenbaum et al., 1997). Increased levels of GCs stimulate the secretion of triglycerides from the liver in the form of vLDL (Bartlett and Gibbons, 1988; Mangiapane and Brindley, 1985) and decrease levels of lipoprotein lipase, which controls the hydrolysis of vLDL. This, in turn, can exaggerate hypertriglyridemia (Taylor and Agius, 1988). Normally, most LDL is removed from the circulation by receptor-mediated endocytosis; however, the binding and degradation of LDL is decreased by dexamethasone, a synthetic GC, resulting in elevations in LDL levels. Excessive LDL levels have been linked to the development of coronary artery disease and stroke. Excessive GC levels also lead to alterations in levels of both insulin and GCs. GCs antagonize insulin's actions: insulin inhibits the secretion of triacylglycerol, cholesterol ester and apolipoproteins, (Brindley et al., 1988) and GCs antagonize these effects by breaking down glycogen and triacylglycerol. In fact, the results from study 3 suggest that many of the adverse effects of a high fat diet may be mediated by increases in circulating GC levels.

Elevated GCs can also directly increase the vulnerability of neurons. A substantial body of evidence suggests that GCs promote hippocampal aging in rodents (Landfield et al., 1981; Sapolsky, 1992; Issa et al., 1990; Bodnoff et al., 1995). In fact, elevated plasma ACTH and B levels are only apparent in aged rats that show spatial memory deficits and not in aged rats showing normal spatial memory function (Issa et al., 1990). The same evidence has recently been found in human populations: aged humans with significant elevations in basal F show reduced hippocampal volume and deficits in hippocampal-dependent memory tasks when compared to same-aged individuals with normal basal F levels(Lupien et al., 1998).

The possibility that dietary factors might provide a clue into the mechanism of glucocorticoid-induced metabolic and neuronal damage has not been extensively investigated. To date, few studies have examined the direct effect of dietary manipulations on hippocampal integrity and learning. A number of groups (Greenwood and Winocur (1990; 1996; Winocur and Greenwood, 1993; 1999) have examined the effects of high-fat diets on tasks measuring short and long-term memory and alternation rule learning. They found that young rats fed high-fat diets that were high in saturated fats were impaired on all tasks tested. They also found that brain membrane composition was affected by fat diets, providing direct evidence that the brain is sensitive to changes in the diet (Greenwood and Winocur, 1996).

Therefore, certain kinds of high-fat diets are associated with chronically elevated GC-titers, a function of the metabolic dependency of animals on the actions of counterregulatory hormones to control blood glucose levels (study 3; Tannenbaum et al., 1997). Further, high fat feeding contributes to insulin resistance in both rats and humans, and alters glucose dynamics. Finally, high fat diets can contribute to cognitive impairments in young animals, possibly through chronically elevated GCs. Since these alterations in HPA axis function and carbohydrate metabolism seem to emerge as a result of chronic elevation in GC levels, the question of the effects of long-term fat-feeding on GC-induced metabolic and neuronal aging was addressed in these studies. We specifically investigated whether chronic high-fat feeding results in basal and stress-induced alterations in HPA activity, whether fat feeding is related to alterations in glucose and insulin dynamics and if elevated fats in the diet are associated with cognitive impairments in aged rats. We specifically examined a spatial task, subserved by the hippocampus, since the magnitude of the increase in HPA activity appears to predict the extent of age-related hippocampal pathology and the cognitive deficits associated with hippocampal damage.

Methods

Subjects

Mid-aged Long-Evans male rats (Charles River, St. Constant, Quebec), approximately 14 months of age, were used for long-term dietary studies. Young Long-Evans male rats, obtained from the same supplier were approximately 5 months of age, and were obtained 1 month prior to the beginning of testing (young rats were tested at 6 months of age). All rats were housed on a 12:12 light-dark cycle (lights on at 0800) and were pair housed throughout the study. Rats were housed in a humidity- and temperature-controlled environment with ad lib access to food (Purina Lab Chow) and water until the beginning of the study. At 16 months of age, animals were randomly assigned to one of two groups, which lasted approximately 7 months: those animals fed a 20% high-fat, termed "Aged Fat" (n=36) or 4% fat, termed "Aged Control" (n=36) diet (see table 7). Young controls, termed "Youngs" (n=15) were used as a comparison group for the effect of age on behavioral, hormonal and metabolic measures and were therefore fed the control (4%) diet for one month. Health of the animals was monitored regularly by a veterinarian and those animals deemed too sick to continue (respiratory difficulties, tumours) were eliminated from the study. Housing and experimental procedures were conducted according to the Canadian Council on Animal Care guidelines and were approved by both the Lady Davis Institute of The Jewish General Hospital and McGill University Animal Care Committees.

Measures taken throughout the study

Body weights and food intake

Body weights (in grams) of the aged animals were taken once per week for the duration of the study (n=36 per group). Food intake was assessed twice per week. Food intake was measured by subtracting uneaten food plus spillage from total food given;

spillage was collected on a diaper placed under the cage. Body weights and food intake were not measured in the young controls.

Variables assessed at the termination of the dietary manipulations

After 7 months exposure to one of the two diets, rats were assessed on a number of behavioral, endocrine and metabolic measures.

Body fat quantification

Body fat was quantified by the water displacement method (Siri, 1961) (n=8 per group). Two independent observers did all quantification procedures. Aged animals were weighed 3 times immediately before testing. The volume of a plastic restrainer was then taken by submerging the restrainer in a large bucket of water and measuring the amount of water displaced (this was done 3 times). An animal was then placed in the restrainer and submerged; the displaced water volume was then measured (this was done 3 times as well). To obtain the volume of the animal, the volume of the restrainer was subtracted from the total volume of restrainer and animal. This was done until less than 10% difference was achieved in 5 independent observations. Once this number was calculated, the density was computed (animal's mass/volume) and entered into the Siri (1961) equation to obtain estimates of body fat: percent fat = 495/(Density-450). Two independent estimates were done on two successive days.

Morris Water Maze

To identify animals with spatial memory impairments, rats were tested in the Morris Water Maze (Aged Fat=16 rats; Aged Control=20 rats; Youngs=15 rats). The maze consisted of a 1.6-m diameter circular pool filled (45-cm depth) with water (22^{0}C) made opaque by the addition of powdered skim milk. A 10-cm2 platform submerged 2 cm below the surface of the water was placed in the centre of one quadrant of the pool. Latencies to find the platform, distance swum, and time and distance in each quadrant were

automatically recorded using a Videomex-V Image Motion System (Columbus Instruments, Columbus OH). Each animal was given three trials per day for five days. At the start of the trial, the animal was placed in the pool at one of four randomly allocated locations. Animals that found the platform within 120 s were allowed to remain on the platform for 10 s; those that did not were manually placed on the platform for the same time period.

On day six, animals were given three probe trials in which the platform was visible (by lowering the water level 2-cm below the top of the platform). These trials were conducted to assess whether the performance of the aged animals could be attributed to visual deficits. Animals received an additional trial where the platform was removed from the pool (30-second duration). This trial allows us to compute swim speeds (to assess motor deficits) where the trial duration is constant across all animals, and also permits an examination of the search strategies used by the animals to locate the platform. Swim patterns were scored as follows. The pool is divided into four equal quadrants (target, i.e the quadrant previously containing the platform), adjacent clockwise, adjacent counter-clockwise, and opposite quadrants), and the percentage of time spent in each quadrant was computed.

Basal ACTH, B and glucose collection

At the end of 7 months exposure to either the high-fat or control diets, basal measures of ACTH and B were taken (n in each group was Aged Fat=14; Aged Control=18; Youngs=9). Blood was sampled from the tail vein (300 ul of blood) taken one hour into the light cycle (0900 h). Sampling was completed in less than one minute following removal from the home cage. Blood samples for B were collected into tubes coated with EDTA placed on ice, and then centrifuged and stored at -20^{0}C until assayed. At the end of the study, animals were rapidly decapitated following less than one minute removal from the home cage (Aged Fat=5; Aged Control=9; Youngs=11). Trunk blood

was collected for later determination of plasma B and blood glucose. Blood collection, centrifugation and storage were as described above.

Stress Sampling

To assess changes in stress-induced ACTH and B following 7 months exposure to the diets, all animals were exposed to a 20 minute period of restraint (Aged Fat=12; Aged Control=16; Youngs=14). Restraint stress was performed between 1000 and 1300 with the use of tubular, plastic restrainers. A blood sample (200 ul) was taken immediately before the rat was placed in the restrainer (termed the "pre" sample) and within 1 minute of removal from the home cage. The animals were restrained for 20 minutes, and blood samples were taken at 10 minutes after the onset of restraint ("-10"). Additional blood samples were obtained at the termination of restraint (termed "0") and at 20, 60 and 120 minutes thereafter. Blood samples for B measurement were collected into tubes coated with EDTA placed on ice, and then centrifuged and stored at -20^{0}C until assayed. Blood samples for ACTH were collected in tubes containing EDTA and aprotinin (Trasylol), centrifuged and stored at -20^{0}C as well.

Glucose Tolerance Test

Animals (n=5 per group)were food-deprived for 12 hours overnight and blood was collected via the tail vein at 08:00 h the following day for measurement of basal plasma glucose levels. Two hours later, animals were injected (i.p.) with 2.5 mg/kg of a 50% glucose solution. Samples were then taken at 15, 30, 60, 120 and 180 minutes post injection. All samples were collected in tubes containing EDTA, centrifuged and stored at -20 ^{0}C until assayed for plasma glucose.

Radioimmunoassays.

Plasma B and ACTH were measured by the radioimmunoassay described in study 3 Plasma glucose concentrations were measured by an automated glucose oxidase method using a Beckman Glucose Analyzer 2 (Beckman Instruments, Fullerton, CA).

Statistical analyses.

The results were analyzed by repeated measures and factorial analyses of variance (ANOVA), and paired and unpaired Student's t-tests. Scheffé post-hoc tests were performed when appropriate. Integrated hormone levels were determined with the trapezoidal rule and the data expressed over time of sampling. $P<0.05$ was considered significant.

Results

Incidence of pathology, mortality and percent body fat are shown in table 10. In general, Aged control and Aged fat-fed rats exhibited the same amount of pathology (tumours, abcesses) and the number of animals who died before the beginning of testing was similar in both groups. High-fat diet fed aged rats had significantly higher body fat ($p<0.05$) when compared to aged control-fed rats following seven months of feeding of the diets.

Body weights over a period of 27 weeks are shown in figure 50. Aged animals fed the high-fat diet had higher body weight throughout the duration of the study and significantly higher body weights during the first 15 weeks on the diet ($p<0.05$), with the exception of the first two weeks on the diets. Body weights remained fairly constant over the 7 months, although both groups showed a modest decrease in body weight over time.

Average food consumption measured daily over a period of 28 weeks is shown in figure 51. Aged rats fed the control diet consistently ate more on average than rats fed the high-fat diet and over time, both groups of rats decreased their food intake when compared to their consumption at the beginning of study. Specifically, at weeks 2,3,4, 17, 19, and 21, control-fed rats ate significantly ($p<0.005$) more than their fat-fed counterparts.

Performance on the Morris Water Maze is shown in figure 52. On days one through four young rats were significantly ($p<0.005$) faster at finding the submerged platform than both the fat- and control-fed aged rats; control and fat-fed aged rats did not differ from each other on days one through four of testing. On day five, however, high-fat fed aged rats had a significantly ($p<0.05$) longer latency to find the platform when compared to control-fed aged and young rats. Young and aged control-fed rats did not differ from each other on day 5. The integrated response on the maze, shown in figure 53, shows that young rats showed an overall shorter latency ($p<0.05$) to find the platform across the five days of testing when compared to aged-fat and -control rats. Aged-fat rats had the highest integrated response. The performance on the elevated platform trials and the trials with the platform removed were similar in all groups.

Figure 54 represents basal levels of plasma B sampled from animals one hour following lights on (AM). Aged animals fed the high-fat diet had significantly ($p<0.05$) higher plasma B in the AM phase of sampling when compared to their control-fed aged counterparts. Control-fed aged rats had slightly lower B levels when compared to young rats but this was not significant.

Plasma ACTH (panel A) and B (panel B) responses before, during and following the termination of a 20-minute period of restraint are shown in figure 55. In panel A, at the termination of restraint (time "0"), both aged groups of rats had significant ($p<0.05$) elevations in plasma ACTH when compared to young rats (figure 55A). This significant ($p<0.05$) elevation persisted at both 20 and 60 minutes following the termination of the

restraint. Finally, at 120 minutes following the termination of restraint, aged control rats showed a significant ($p<0.05$) elevation in plasma ACTH when compared to young rats. In figure 55 B, plasma B levels prior to the onset of restraint were significantly ($p<0.05$) elevated in aged animals fed the high-fat diet compared to both aged control-fed rats and youngs. At 10 minutes into the stress, both high-fat fed and young animals had significantly ($p<0.05$) elevated B levels when compared to control-fed aged rats. Aged fat-fed animals had sustained ($p<0.05$) elevations in plasma B when compared to both aged control-fed and young rats at 60 minutes following the termination of restraint, while at 120 minutes following the restraint, both fat-fed and control-fed aged animals had elevated B when compared to young rats. The integrated plasma ACTH and B response to restraint are shown in figure 56A and 56B respectively. Aged fat-fed rats had a higher overall integrated ACTH and B response when compared to aged control-fed and young rats.

The response to an i.p. injection of glucose is shown in figure 57. The "pre" value is an estimate of basal blood glucose levels. Young animals had significantly elevated blood glucose when compared to aged fat-fed and control-fed rats ($p<0.05$) at the pre time point. At 15 minutes following the injection of glucose, both young and control-fed aged rats showed significant ($p<0.05$) elevations in blood glucose when compared to aged fat-fed rats. Aged fat-fed rats continued to show a blunted response to glucose at 30 and 60 minutes following the injection of glucose. The integrated response to the i.p. glucose injection is shown in figure 58. Aged fat-fed rats had a lower integrated response when compared to both aged control-fed and young rats. Control-fed aged and young rats had a similar integrated response.

Figure 59 shows plasma B (panel A) and blood glucose (panel B) obtained from trunk blood at the time of sacrifice. In panel A, only aged control-fed rats had significantly elevated plasma B when compared to young rats. Aged fat-fed rats had elevated plasma B compared to young rats, but this was not significant. Blood glucose measured from trunk

blood at the time of sacrifice is shown in figure 59B. Young animals had significantly elevated blood glucose levels ($p<0.05$) when compared to both groups of aged rats.

Table 10. Percent body fat, incidences of pathology and number of deaths prior to testing in aged control-fed and aged fat-fed rats.

Group	% Body Fat	Incidences of Pathology	# Deaths Prior to Testing
Aged Fat	44.2 %	7	14
Aged Ctl	15.5 %	7	11

& Aged Fat significantly different from Aged Ctls.

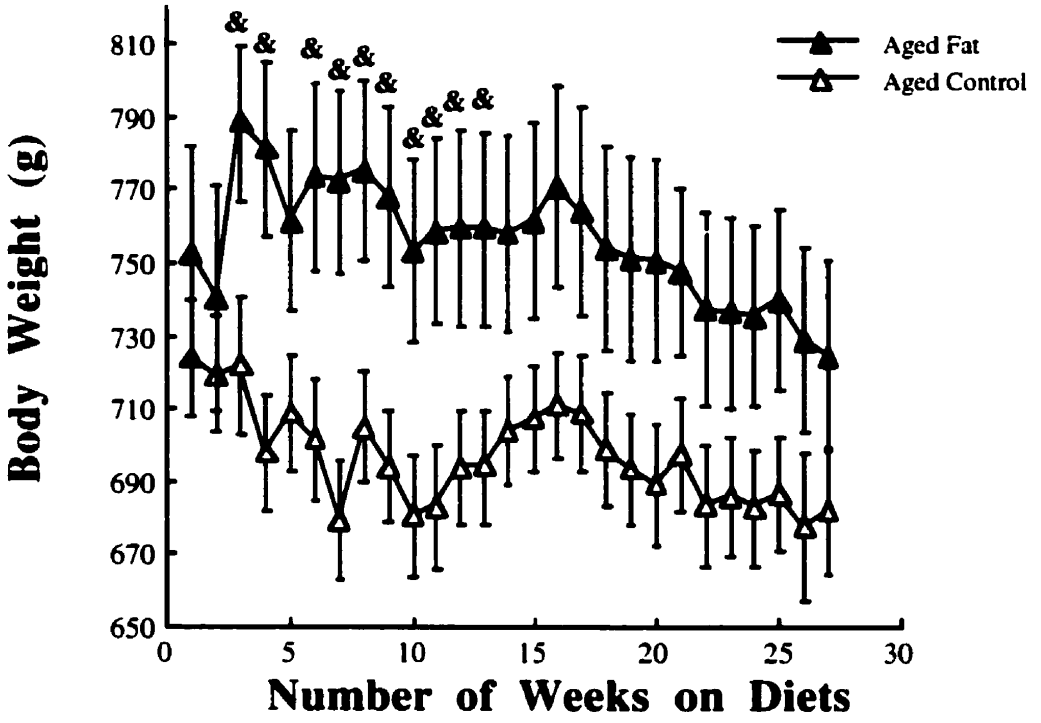

Figure 50. Mean (± SEM) body weight (grams) of aged animals exposed to either a high-fat (Aged Fat) (n=36) or control (Aged Control) (n=36) diet over 27 weeks. Weights were taken twice a week and averaged across groups. & Aged Fat significantly different from Aged Control.

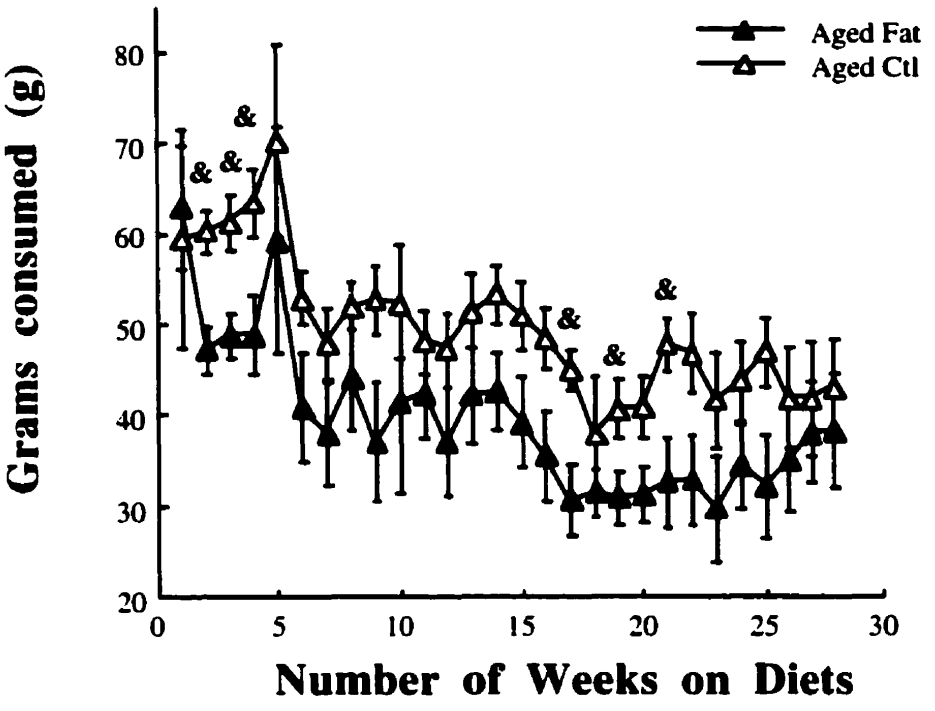

Figure 51. Mean (± SEM) daily food intake (grams) of aged animals exposed to either a high-fat (Aged Fat) (n=36) or control (Aged Control) (n=36) diet over 27 weeks. Food was weighed daily and the average consumption per week/per day is shown. & Aged Fat significantly different from Aged Control.

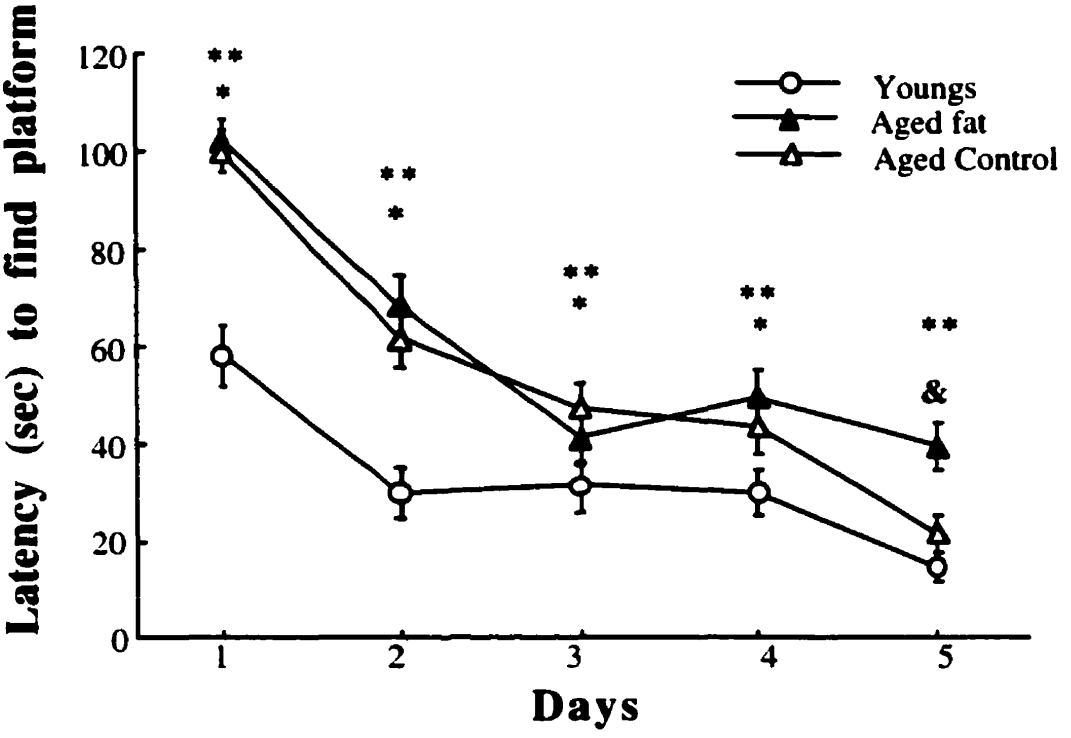

Figure 52. Mean (± SEM) latency (seconds) to locate a submerged platform on the Morris Water Maze task in aged animals exposed to either a high-fat (Aged Fat) (n=17) or control (Aged Control) (n=20) diet and control-fed young rats (Youngs) (n=15). * Youngs significantly different from Aged Control; ** Youngs significantly different from Aged Fat; & Aged Fat significantly different from Aged Control.

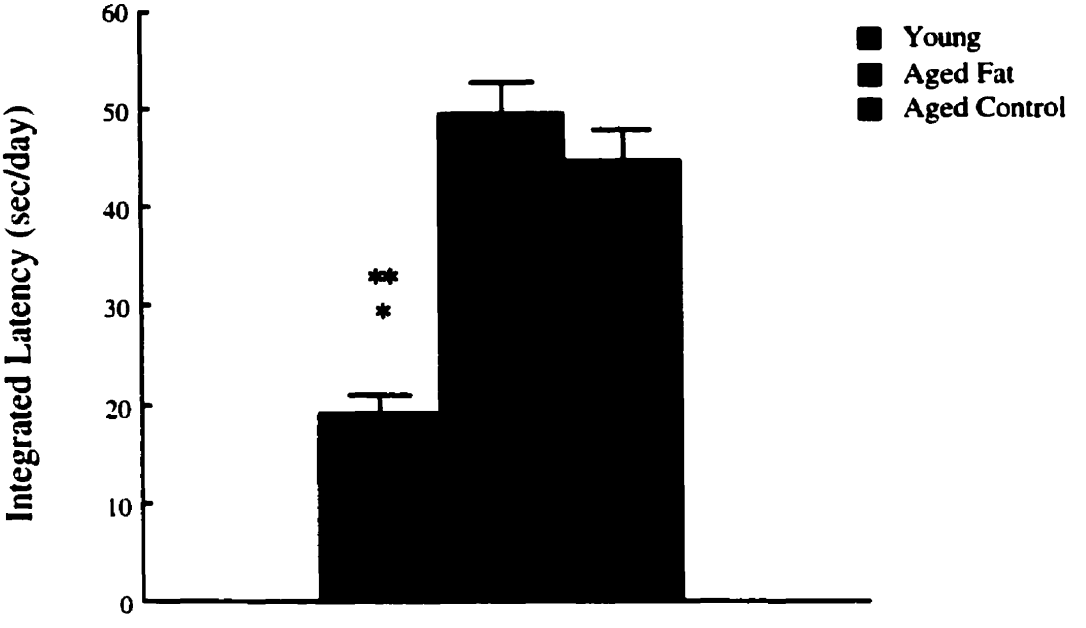

Figure 53. Mean (± SEM) integrated latency to locate a submerged platform over 5 days of testing on the Morris Water Maze levels in aged animals exposed to either a high fat (Aged Fat) (n=17) or control (Aged Control) (n=20) diet and control-fed young rats (Youngs) (n=15). * Aged Control significantly different from Youngs; ** Aged Fat significantly different from Youngs.

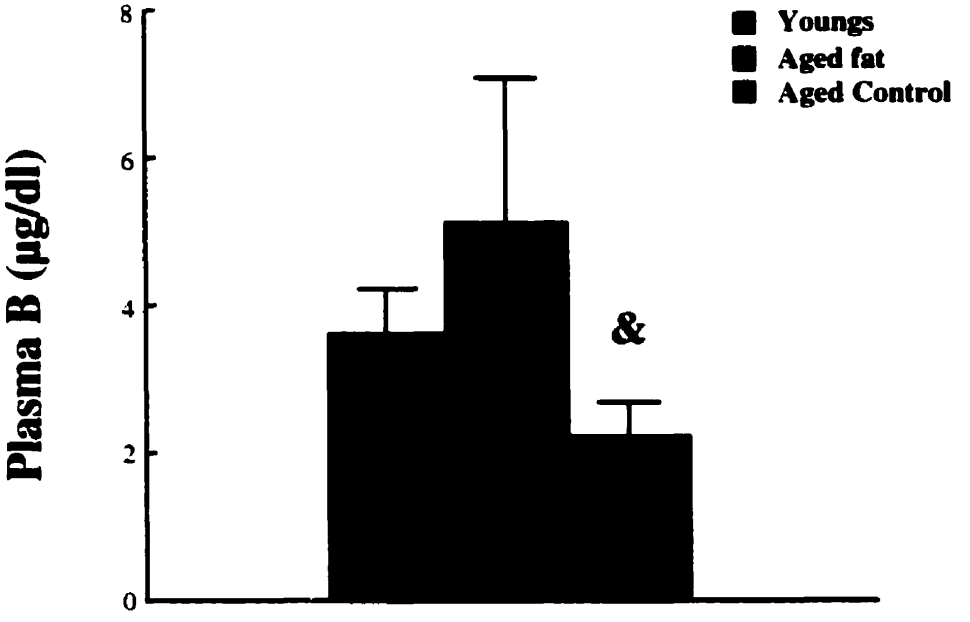

Figure 54. Mean (± SEM) plasma B levels in aged animals exposed to either a high fat (Aged Fat) (n=14) or control (Aged Control) (n=18) diet and control-fed young rats (Youngs) (n=9) sampled one hour after lights on (AM). & Aged Fats significantly different from Aged Controls.

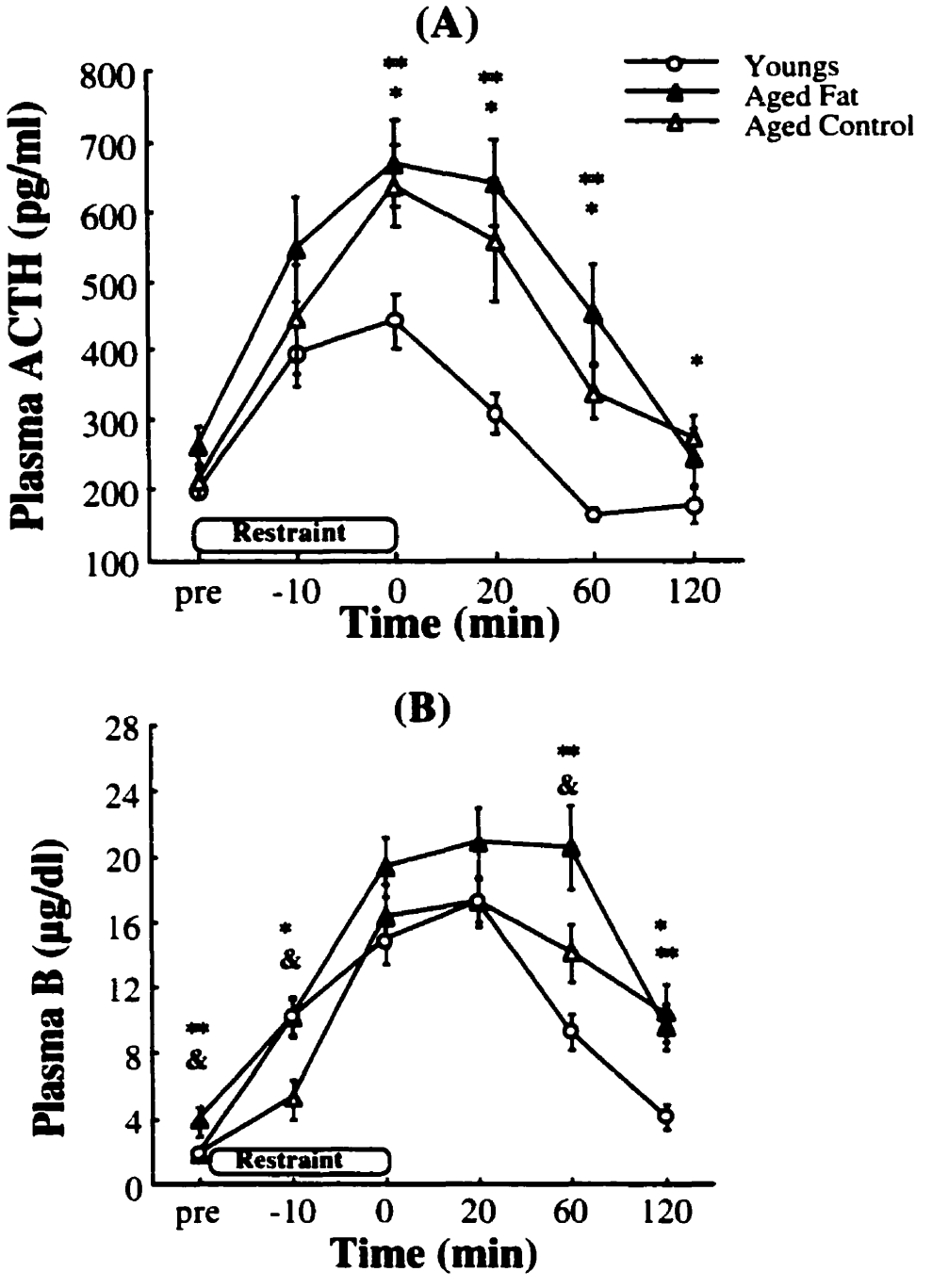

Figure 55. Mean (± SEM) plasma ACTH (panel A) and corticosterone (panel B) levels in aged animals exposed to either a high fat (Aged Fat) (n= 12) or control (Aged Control) (n=16) diet and control-fed young rats (Youngs) (n=14) immediately before (PRE), during (-10), at the termination of (0) and at numerous points (20, 60 and 120 min) following a twenty minute period of restraint. & Aged Fats significantly different from Aged Controls; ** Aged Fats significantly different from Youngs; * Aged Controls significantly different from Youngs.

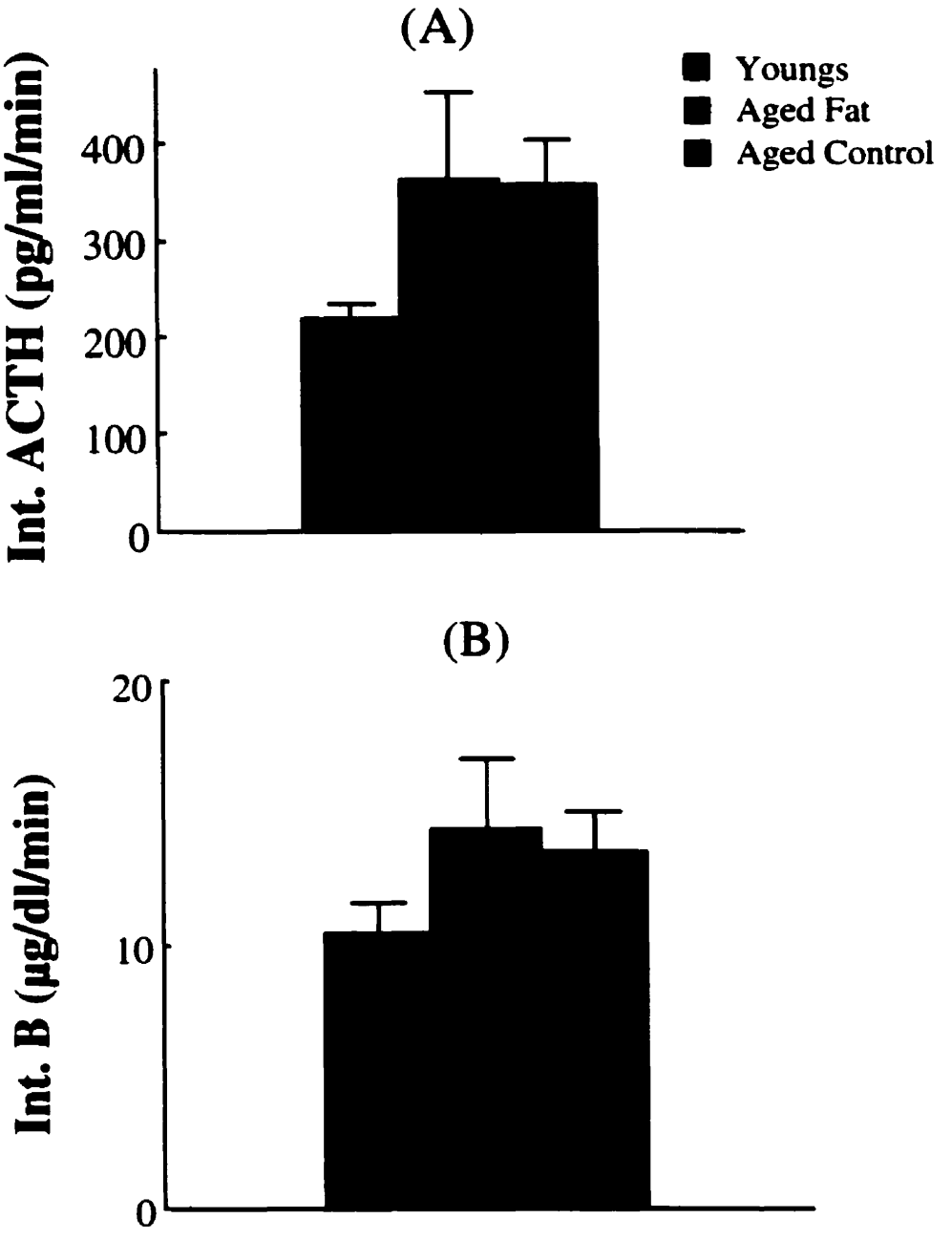

Figure 56. Mean (± SEM) integrated levels of plasma ACTH (A) and corticosterone (B) in aged rats fed either a high-fat (Aged Fat) (n=12) or control (Aged Control) (n=16) diet and young control-fed rats (Youngs) (n=14) prior to, in response to and following a twenty minute period of restraint .

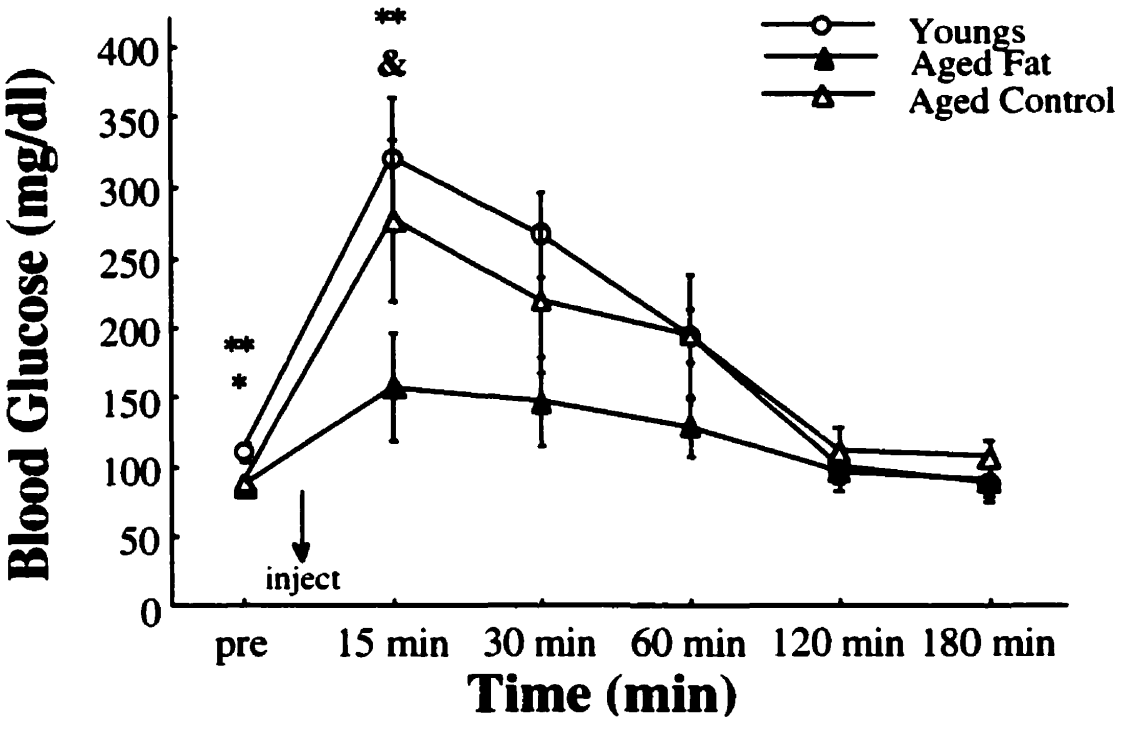

Figure 57. Mean (± SEM) blood glucose response prior to (pre) and at numerous time points following a 2.5g/kg i.p. injection of 50% dextrose in aged animals exposed to either a high fat (Aged Fat) (n=5) or control (Aged Control) (n=5) diet and control-fed young rats (Youngs) (n=5). Animals were food deprived 12 hours prior to the pre sample. & Aged Fat significantly different from Aged Control; ** Aged Fat significantly different from Youngs; * Aged Control significantly different from Youngs.

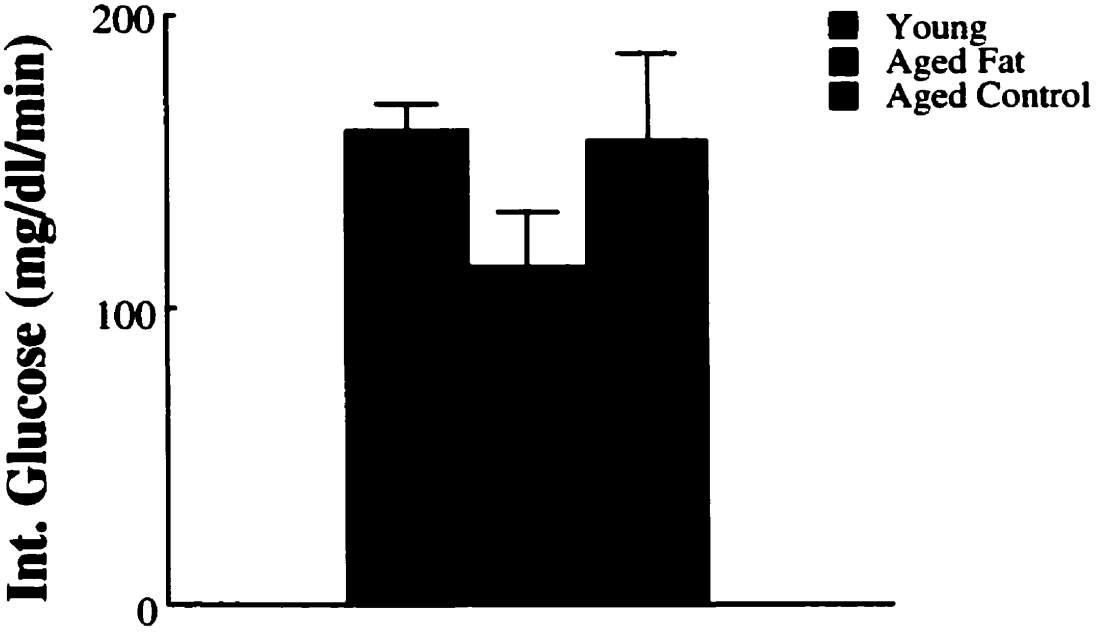

Figure 58. Mean (± SEM) integrated blood glucose levels in aged rats fed either a fat (Aged Fat) (n=5) or control (Aged Control) (n=5) diet and control-fed young rats (Youngs) (n=5). Integrated levels were calculated from blood glucose measures obtained immediately prior to and at numerous time points following a 2.5g/kg i.p. injection of 50% dextrose.

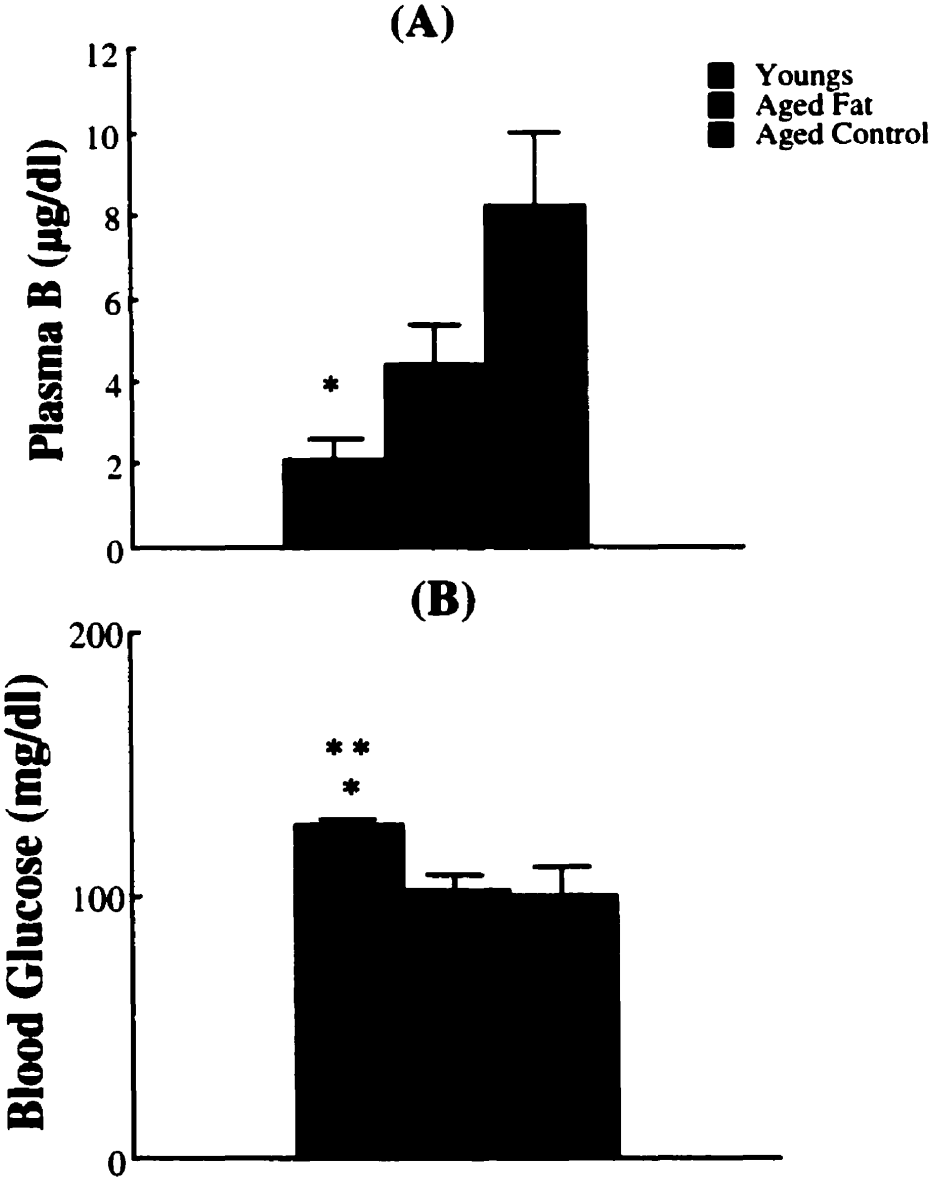

Figure 59. Plasma (mean ± SEM) B (A) and blood glucose (B) at time of sacrifice obtained from trunk blood of aged rats fed either a high fat (Aged fat) (n=5) or control (Aged Control) (n=9) diet and control-fed young (Youngs) (n=11) rats. *Aged Control significantly different from Youngs; ** Aged Fat significantly different from Youngs.

Discussion

These data suggest that chronic high-fat feeding increases body fat content, and elevates body weight with diminished food intake. Prolonged high-fat feeding elevates basal and stress-induced HPA axis functioning and lowers basal and stimulated blood glucose levels. The data also suggest that elevated fat in the diet causes subtle impairments in the acquisition of a spatial task.

Aged animals fed a high-fat diet eat significantly less food, yet have a higher percent of body fat and weigh significantly more. Aged animals fed the high-fat diet had the highest accumulation of body fat in the abdominal area. This is of considerable importance since a number of recent studies suggest that body mass index and the amount of body fat accumulated may be a stronger predictor for coronary artery disease, stroke, cancer and diabetes than body weight itself (Bouchard et al., 1993). In general, results from numerous studies suggest that an accumulation of fat in the upper part of the body is a risk factor for NIDDM and coronary heart disease (Laarson et al., 1984; Bjorntorp, 1988). Clinical studies in obese individuals suggest that the amount of abdominal fat correlates strongest with plasma lipid and cortisol levels (Sparrow et al., 1986). In addition, a high waist to hip ratio (an accumulation of fat in the abdominal area, also known as android obesity) is associated with high plasma triglyceride levels, elevated low high-density lipoprotein cholesterol concentrations and increased plasma cortisol.

On average, high-fat fed animals consumed significantly less food. This may be due to the higher caloric density of the high fat diet (4.8Kcal/gram versus 4.0 Kcal/gram for the control diet). Therefore, even though rats may be consuming fewer grams of the high-fat diet, the total calories consumed may be higher. While leptin was not measured in this study it is possible that these levels are altered in the high-fat fed aged rats. In general, leptin is thought to act as a feedback hormone that signals the amount of fat stores in the body to the hypothalamus. The hypothalamus in turn effects changes in appetite and

metabolism to regulate energy stores (Tan et al., 1998). In the obese fa/fa rat circulating leptin levels are high, but there is a mutation in the leptin receptor gene in the hypothalamus. Therefore leptin is unable to reach the brain and decrease NPY levels, which are also elevated in the genetically obese rat (Rohner-Jeanrenaud and Jeanrenaud, 1996). The increased NPY levels may then stimulate the HPA axis to secrete more B and the pancreas to secrete more insulin. The combined effects of B and insulin include excessive fat accretion and this condition then further increases leptin secretion. The other possibility is that the aged animals fed the high-fat diet are leptin-deficient and as a result, their level of NPY in the brain is elevated relative to their control-fed counterparts. Increased NPY levels will then result in increased HPA axis activity and increased fat deposition.

A number of studies have examined the direct effect of dietary manipulations on hippocampal dependent learning. Greenwood and Winocur (1990; 1993; 1996) have examined the effects of high-fat diets on tasks measuring short and long-term memory and alternation rule learning. They found that young rats fed the high-fat diets that were high in saturated fats were impaired on all tasks tested. They also found that brain membrane composition was affected by fat diets(Greenwood and Winocur, 1996). In the studies by Greenwood and Winocur, only rats fed the high saturated fat diet were impaired on cognitive tasks; those fed unsaturated fat diets were not impaired on these tasks. Grundy et al. (1982) found that diets that are high in polyunsaturated and monounsaturated fats (vegetable oils) actually lower blood cholesterol and that diets high in polyunsaturated fats have been more effective than low-fat, high carbohydrate diets in lowering total serum cholesterol as well as the incidence of coronary artery disease. Monounsaturated fats elevate plasma HDL without affecting LDL levels (Mensink et al., 1992). There is also evidence for the contrary: unsaturated fats have been shown to raise LDL levels, increase lipoprotein levels and elevate triglycerides (Katan et al., 1995); these unsaturated fats were of the trans unsaturated fat family, suggesting that the source of fat is critical when

interpreting the results obtained from studies using high-fat diets, as some fats may be protective, while others may be damaging. A number of studies have shown that caloric restriction appears to reduce or retard the occurrence of many physiological alterations associated with aging, including the sparing of age-associated impairments on the acquisition of the Morris Water Maze (Means et al., 1993; Bellush et al., 1996). These studies provide direct evidence that the brain is sensitive to changes in the diet and that diets high in fat and calories may cause cognitive impairments, either by directly affecting brain morphology or by indirectly elevating the level of particular hormones that are known to alter neuron function and number. While our aged fat-fed rats showed only modest impairments on the Morris water maze, the impairment may be a result of either the fat in the diet itself or the increased caloric consumption. Since our source of fat was not saturated, the cognitive impairments seen were milder than those found after prolonged saturated fat feeding

Aged rats fed the high-fat diet showed elevations in AM basal B levels and an increased B response to an acute stressor. Stress-induced alterations in ACTH were evident in both groups of aged rats when compared to young control-fed rats, and aged rats fed the high-fat diet showed an overall higher stress-induced ACTH release when compared to aged rats fed the control diet. This finding is interesting for three reasons. First, in young rats, high-fat diets cause alterations in both basal and stress-induced HPA axis function, presumably through increased free fatty acid (FFA) levels (Tannenbaum et al., 1997). Because GCs tend to stimulate lipolysis, elevations in B may further stimulate the production of FFA, which are already elevated due to the fat content of the diet. This results in an increase in both B and FFA, which in combination represents a tenuous metabolic state. Second, young and aged animals fed the high-fat diet show an HPA profile that is similar to animals that are chronically stressed. Chronically stressed rats have higher basal plasma B in the AM and an elevated HPA response to a novel stressor (Scribner et al., 1991). Therefore one could consider a high-fat diet as a type of chronic stressor;

chronic activation of the HPA axis is associated with hippocampal neuron loss, loss of glucocorticoid receptors, decreased LTP and the emergence of cognitive deficits in the aged rat (Landfield et al., 1978; Sapolsky, 1985; Issa et al., 1990; Bodnoff et al., 1995). Third, aged animals that are selected for spatial memory deficits show increased HPA activity and the occurrence of neuron loss and decreased glucocorticoid receptor levels in the hippocampus, an area known to be critical for spatial learning (Issa et al., 1990). Further the magnitude of cognitive impairments is related to the magnitude of HPA dysregulation. While both groups of aged animals showed impairments in the acquisition of the maze, aged animals fed the high-fat diet had the highest overall latency to find the platform, significantly elevated basal plasma B in the AM and the highest integrated stress-induced ACTH and B levels when compared to aged animals fed the control diet and youngs.

Another possible mechanism for the small cognitive impairment seen in aged animals fed the high-fat diet is lower basal and stimulated glucose levels. A number of studies have examined the memory-improving action of glucose in both aged and young animals (Korol and Gold, 1998; Messier and Gagnon, 1996; White, 1991). Glucose has been found to improve encoding and in some cases, retrieval, in a variety of tasks (Messier and Gagnon, 1996) and can attenuate novelty-induced anxiety (Kopf and Barrati, 1996). Here, aged rats fed the high-fat diet had lower basal glucose (both at the Pre time point and at sacrifice) and a blunted glucose response to glucose challenge when compared to both control-fed aged rats and youngs. Aged rats fed the control diet also exhibited a lower basal and stimulated glucose response compared to youngs but fat-fed aged rats showed a much more blunted response to glucose challenge. Therefore one possible mechanism for the aged animals' impaired performance on the Morris Water maze is a diminished overall glucose output.. In a recent report by Messier (1997), animals that received an injection of glucose spent more time exploring in an open field and explored a novel object for a greater amount of time (when compared to a previously seen object). This suggests that glucose can retroactively improve the memory for a previously observed object. In a study by

Kopf and Baratti (1996), mice were allowed to explore a novel environment, provided by an open field on two separate occasions within 24 hours. The difference in the exploratory activity between the first (training) and second (testing) exposures was taken as an index of retention of this habituation task. Postraining i.p. administration of glucose enhanced retention, suggesting that glucose can modulate memory storage of one form of learning elicited by stimuli that are repeatedly presented. Glucose administration also has beneficial effects on memory. When administered just before or after training, glucose enhances memory for recent experiences in healthy elderly people (Craft et al. 1993; Hall et al., 1989; Manning et al., 1990; 1992; 1997) as well as in rodents (Wenk, 1989; White, 1991; Gold, 1991). Li et al. (1998) found that a pre-training i.p. glucose injection enhanced memory performance in both a passive avoidance task and on the Morris water maze. A recent report by Manning et al. (1998) suggests that when healthy elderly human subjects are administered glucose immediately before being tested on recall for a passage of text that was read 24 hours earlier, memory storage and retrieval are enhanced. Craft et al. (1993) demonstrated that individuals with dementia of the Alzheimer's type showed memory facilitation after administration of glucose. Glucose has been found to improve encoding and in some cases, retrieval, in a variety of tasks (Messier and Gagnon, 1996).

The mechanism of glucose's action on cognition includes both peripheral and central effects. Blocking liver efferents has been shown to abolish the effect of glucose on memory (White, 1991) and administration of fructose, which does not cross the blood brain barrier, produces similar memory-enhancing effects as glucose (Messier and White, 1987; Rodriguez et al., 1994). Peripheral actions may require a central neural signal, however, that is produced when glucose is carried into cells via glucose transporter mechanisms (Jay et al., 1990). Glucose easily enters the brain and is its main metabolic fuel (Sieber and Traystman, 1992). Recent microdyalisis experiments showed that when blood glucose concentrations were raised by 300% by a steady infusion of glucose, there was a 200% increase of extracellular glucose in the hippocampus (Harada et al., 1993).

Glucose could act as an additional metabolic substrate and be used to supply energy. A number of groups have also positively correlated circulating plasma glucose and the rate of 2-deoxyglucose disappearance with local cerebral glucose utilization (LCGU), suggesting that there may be specific effects of insulin on brain glucose utilization (Vissing et al., 1996). Extremely high or low levels of insulin could affect glucose utilization in those brain regions where insulin receptors are densely represented, namely the hippocampus, the olfactory bulb, and the hypothalamus (Craft et al., 1993). More specifically, glucose has been shown to attenuate the reduction of acetylcholine (ACh) content in the hippocampus produced by atropine injections and glucose increases extracellular ACh following scopolamine injections. Glucose also gets taken into cholinergic presynaptic terminals and transformed into an ACh precursor (Ricny et al., 1992; Durkin et al., 1992). A recent report by Ragozzino et al (1998) demonstrated that a unilateral infusion of glucose into the hippocampal formation potentiated the increase in hippocampal ACh output during spontaneous alternation testing. In marked contrast, when administered to rats at rest, glucose infusions did not modify hippocampal ACh output in the ispilateral or contralateral side, suggesting that glucose may only increase ACh output under conditions in which the activity of the cholinergic neurons is "significantly" altered (Ragozzino et al., 1998).

Other systems may be affected by glucose administration as well. When the dopamine (D2) receptor agonist quinpirole improves performance on a radial maze task, blood glucose levels are elevated. This effect of quinpirole on glucose was due to the release of catecholamine from the adrenal medulla produced by the activation of central D2 receptors. When the D1 agonist SKF 38393 is given there is no concomitant rise in glucose nor is there any improvement on the radial maze task. Glucose can also attenuate the amnesic effects of morphine and that this attenuation may be related to the reversal of morphine's action on hippocampal cholinergic function. Talley et al. (1999) have recently shown that peripherally administered glucose attenuated morphine-induced deficits in spontaneous alternation. Glucose has been shown to attenuate the amnesia produced by

post-training scopolamine in various learning tasks (Messier et al., 1990; Stone et al., 1991). In the study by Li et al. (1998) described above, one potential mechanism of glucose's action is on acidic fibroblast growth factor (aFGF), which serves to suppress cells from degeneration and death, specifically in the hippocampus. In their study, glucose-induced memory facilitation effects were abolished by an icv injection of anti-acidic-FGF antibody.

Numerous studies have suggested that prolonged fat-feeding may render an individual at risk for developing many chronic disease, such as cancers, heart disease and NIDDM. It is clear from this study that aged animals fed a high-fat diet are not diabetic. .One possible explanation for this is that the risk for the development of NIDDM is partly dependent on the amount of fat in the diet. The critical feature is the type of fat consumed. A number of studies have shown that monounsaturated and polyunsaturated fatty acids may not necessarily lead to alterations in insulin action; in fact these fatty acids may improve indexes of glucose tolerance in NIDDM (Low et al., 1996; Wilkes et al., 1998). In addition the amount of adiposity in the aged animals fed the high-fat diet may have played a role in the uptake of glucose: the larger fat mass in these rats may account for an absolute increase in the amount of glucose disposed and may help to preserve glucose tolerance. Conversely, the glucose injected may have been absorbed in the fat tissue.

In sum, despite an overall decrease in food intake, these data suggest that prolonged fat feeding increases adiposity and body weight. These alterations in body mass may then be associated with elevations in basal and stress-induced ACTH and B and a decrease in basal and stimulated glucose. Alterations in ACTH, B and glucose may then act together, with other systems, such as NPY or leptin or may even be acting alone in mildly impairing the acquisition of the Morris water maze, possibly through a diminishment of hippocampal neuronal integrity. These data suggest that chronic high-fat consumption

may cause aberrations in HPA axis functioning, which may be associated with perturbations in glucoregulatory processes that are integral to hippocampal integrity.

Study 5 Contributors of Individual Variation in Cortisol Profiles in Healthy Elderlies: Dietary Factors

In study 4, prolonged high-fat feeding resulted in higher basal and stress-induced levels of B, suggesting fat-fed aged rats had a higher cumulative exposure to GCs over the lifespan. Again, this emphasizes that aging, per se, is subject to numerous influences and is not an inevitability. In fact, there is considerable variation in hypothalamic-pituitary-adrenal (HPA) and cognitive function in both aged humans and rats. In rats, increased HPA activity is not necessarily a consequence of aging, but is readily apparent in rats selected for spatial memory deficits (a hippocampal sensitive task) versus cognitively-unimpaired rats. Issa et al. (1990) examined HPA function in aged rats that were tested for spatial learning impairments. They found increased basal and stress-induced HPA activity in cognitively-impaired rats compared to either cognitively-unimpaired or young, adult controls. The presence of the cognitive impairments and concomitant increased HPA activity was associated with hippocampal neuron loss. Bodnoff et al (1995) demonstrated significantly reduced hippocampal synaptic plasticity (dampened prime-burst potentiation) and impaired acquisition of a spatial task in animals treated with both medium- and high-levels of corticosterone (B) from mid-age onward, suggesting that cognitive impairments emerge, in part as a result of long-term exposure to GCs. These data are consistent with previous *in vivo* and *in vivo* studies correlating decreased prime burst potentiation (PBB) or long-term-potentiation (LTP) with age-related memory deficits (Landfield and Lynch, 1977; Landfield et al., 1978, Barnes, 1979, Moore et al., 1993). Therefore these data suggest that both the number and functional plasticity of hippocampal neurons can be

affected by chronic elevations in GCs. Furthermore, these studies suggest that while brain aging can be accelerated by increased levels of adrenal hormones, the variation in adrenal hormone levels in aged rodents is correlated with the variation in the rate of brain aging (Landfield et al., 1978; 1981).

Similar work has been carried out in humans. Studies in humans show that basal cortisol (F) levels generally do not change with age in healthy subjects but are significantly elevated in the context of Alzheimer's disease and depression. Alzheimer's patients showing progressive hippocampal degeneration show an inverse relationship between F levels and hippocampal volume and a positive relationship between the extent of cognitive decline and cortisol levels (Davis et al., 1986). In Cushing's syndrome, hippocampal is negatively correlated with plasma cortisol levels and positively correlated with verbal memory test scores (Starkman et al., 1992). Previous work in our laboratory (Lupien et al. 1994) examined basal cortisol levels in a group of healthy elderly subjects and identified the existence of three subgroups: those with increasing, decreasing or stable cortisol levels with years. These subjects were identified based on a simple regression analysis, with year as the independent variable and cortisol (integrated 24 hour) as the dependent. The slope of the line represented the change in cortisol with years. Current cortisol levels were considered as the mean of the 24-hour sample taken on the last year of the study. Lupien et al. (1994) found that those subjects showing increasing cortisol with years and currently elevated cortisol levels were impaired on tasks involving explicit memory, while those subjects who showed decreasing cortisol levels with years performed as well as young, healthy volunteers with regard to cognitive performance (Lupien et al., 1994). What makes the cognitive data so intriguing is that subjects who showed increasing cortisol with years were specifically impaired on cognitive tasks that are subserved by the hippocampus, namely explicit and declarative memory. Lupien et al. (1998) then examined whether prolonged cortisol elevations and memory impairment would correlate with a significant decrease in hippocampal volume. The authors reported that indeed, prolonged elevation of

cortisol correlates with hippocampal damage and related cognitive deficits in a subset of the same sample of healthy elderlies (Lupien et al., 1998). Currently high and rising cortisol levels over five years were linked to a 14% decrease in hippocampal volume compared to age-matched controls whose cortisol levels were lower and within the normal range of young subjects.

The question that follows from these studies is what are some of the potential sources of this variation in cortisol levels among a population of healthy elderlies? The first obvious candidate would be factors such as lifelong exposure to stress, chronic anxiety and depression and lack of social support. When the same population of healthy elderlies were assessed on the SCL-90, a self-rating questionnaire that measures obsessions/compulsions, fears, anxieties and depression, Lupien et al. (1996) found a positive correlation between cortisol slope and the obsessive/compulsive subscale. These data suggest that aged subjects who develop a trend towards high cortisol levels later in life show more subjective fears than those showing moderate or decreasing cortisol levels. The limitation here, however, is that results of the SCL-90 might simply represent the normal fears associated with aging and may not be representative of life-long stressors. In addition, retrospective studies are limited by how much subjects can recall during their lives, the accuracy of what they recall, and the definition of what they consider a "stressor". Therefore, a more recent assessment of life events and lifestyle might better represent some of the contributing factors for the emergence of their cortisol profile.

Another factor that might contribute to an individual's cortisol profile is diet. Diets that are high in fats are associated with increases in circulating corticosterone (see thesis studies 3 and 4; Castonguay, 1991; Tannenbaum et al., 1997; Kamara et al., 1998). Excessively high levels of GCs can cause a state of insulin resistance, which results in hyperinsulinemia and can cause further increases in circulating GCs. This combination of conditions also emerges as a direct result of high amounts of fat in the diet and is often

accompanied by increased lipolysis and increased plasma levels of free fatty acids (Reaven, 1988; Tannenbaum et al., 1997). While elevations in glucose and insulin have been associated with aging, current theories of aging suggest that dietary factors, and not age itself are the best predictors for the development of abberations in glucose metabolism, including insulin resistance, insulin insensitivity and glucose intolerance. Many studies have also found that dietary and exercise modifications may substantially blunt the emergence of carbohydrate intolerance and insulin resistance with increasing age (Rowe and Kahn, 1987). Zavaroni et al. (1986) evaluated the relative contributions of obesity, physical activity and family history of diabetes to age-related increases in glucose and insulin levels after an oral glucose tolerance test in factory workers aged 22 to 73 years. The initially strong correlation between age and both post-prandial glucose and insulin levels became significantly weaker when the effects of exercise and diet were taken into account. Barnard's group (Barnard et al., 1995) found that the diet, not aging was responsible for causing skeletal insulin resistance in aged rats. No significant differences were observed between 6- and 24-month old rats raised on a low-fat, high-carbohydrate diet on serum glucose and insulin or for basal or insulin-stimulated glucose transport, providing strong support that aging per se does not lead to insulin resistance. When 24-month-old animals raised on the low-fat, high-carbohydrate diet were compared to rats raised on a high-fat diet, major differences were observed: fasting serum insulin was significantly higher in the high-fat diet group and insulin-stimulated glucose transport was significantly reduced (Barnard et al., 1995). Therefore perturbations in glucose regulation do not simply emerge as a function of age, but rather as a function of individual life-long habits.

High-fat diets not only affect metabolic indices of aging; a number of studies suggest that high-fat diets can cause cognitive impairments in adult rats (Greenwood and Winocur, 1990; 1993; 1996). While these authors did not measure GC levels in these animals, it has been shown that both short- and long-term fat feeding can cause reduced

insulin sensitivity which may can result in a compensatory rise in plasma corticosterone levels (Tannenbaum et al., 1997; see study 1) and may, in turn, render hippocampal neurons vulnerable to GC-induced damage. In addition to the issue of fat content in the diet, a substantial body of evidence suggests that restricting caloric intake over the lifespan has protective effects on the functional integrity of hippocampal neurons, and caloric restriction can, in part, prevent the brain from becoming vulnerable as it ages. Therefore there is a reciprocal relationship between an individual's dietary lifestyle and cortisol profile, which can render an individual at risk for the development of metabolic and even cognitive dysfunction in aging.

There is clear evidence for variation in both cortisol and insulin/glucose levels in healthy elderly populations. Since dietary variables seem to affect both F and glucose dynamics (studies 3 and 4; Tannenbaum et al., 1997), then it would seem reasonable to propose that one of the potential sources of variation in cortisol levels might be dietary variables themselves. Therefore we hypothesized that individuals who showed a higher consumption of fat, carbohydrate and calories over a 30-day period would also have elevations in salivary F taken over the same period of time. We chose at-home salivary F sampling as a non-invasive method to measure the natural variation in F. This measure of salivary F reflects the dynamic measures in plasma F represented by the plasma F slope and by current plasma F levels.

Methods

Subjects

Eight elderly, French-speaking subjects (three men and five women) ranging from 65-85 were included in our group. Two more men were in the study but had to be omitted since one subject suffered a heart attack during the 30 day saliva study and the other

subject's saliva samples were destroyed in transport. Men and women were of equivalent age (men, 72.0 ± 4; women, 72.2 ± 3.2 years) and education level and were part of a broader population that were being assessed on the effects of age on circadian hormonal rhythms at the Douglas Hospital Research Center in Montreal (Lupien et al., 1994, 1997). The status of the subjects was determined by a complete physical examination, including body weight, blood pressure, electrocardiogram, electroencephalogram, computerized axial tomography scan, a battery of laboratory tests for determination of kidney, liver and thyroid functions, hemogram, vitamin B12, folate levels, blood glucose, blood cholesterol, blood HDL/LDL ratio, triglycerides, as well as a neuropsychological assessment. Only subjects with a normal range of results were included in the population. Informed consent was obtained from all subjects. For purposes of this study, only the most recent plasma levels (from the 1996 physical examination) of cholesterol, HDL, LDL, glucose, triglycerides and 24-hour plasma F were examined in this study. Ratios of total to HDL and LDL to HDL cholesterol were calculated as well, as these represent more accurate risk factors for CAD than do the levels of each particle or levels of total cholesterol.

Plasma F Characterization (once per year)

Measures of F levels were obtained once per year, beginning in late 1987. Some subjects participated throughout the entire course of the study while others joined it at various times between the first and last year. This gave rise to a population composed of individuals having been measured twice or more. Each subject had been seen during the same month for each consecutive year.

All subjects were sampled for a 24 hour period using an indwelling forearm catheter kept patent with a 0.3% heparin saline solution. Throughout the course of sampling, illumination was maintained at 300 lux during the "daytime" (0700 to 2300) and at 50 lux during the "nighttime" (2300 to 0700). Blood samples were taken each hour, centrifuged at 2500 rpm for 10 minutes at 0-4^{0}C, frozen and stored at -20^{0}C until assayed. Cortisol

levels in plasma were determined using an RIA kit (New England Nuclear Corp., Boston, MA) with cortisol ^{125}I as tracer and a cortisol antibody that binds moderately with corticosterone (15%), but less than 4% with 11-deoxycortisol, 17a-hydroxyprogesterone, prednisone, cortisone, or progesterone. The minimum sensitivity of the assay is 200pg/100ml, with inter- and intraasaay variability of 5% and 3.5% respectively.

Three cortisol measures were used for each subject. The cortisol slope was first calculated using a simple regression analysis and served as an estimate of the "cortisol history" of subjects since the magnitude and the direction of the slope determine the importance of cortisol augmentation or decline with years. The longitudinal measures of each subject were thereafter averaged across years, giving another estimate of the cortisol status that takes into account variability of cortisol levels across years. Finally plasma cortisol levels taken on the last year of the study was used in order to assess "static" cortisol levels. Subjects were then classified in the following way: the first group who presented with a positive slope and elevated basal cortisol levels on the last year of the study and were termed "increasing". The second group presented a positive slope but had moderate cortisol levels on the last year of the study and were termed "stable" and finally a third group presenting an overall negative slope with moderate current cortisol levels were termed "decreasing." For the 30 day salivary cortisol study, we grouped the individuals who showed increasing plasma cortisol ("increasing") into one group and those showing stable or decreasing cortisol into another group when we compared the saliva cortisol levels and dietary intake. We combined the stable and decreasing group due to a small sample size in each of those respective groups. For purposes of clarity, we used the 24-hour plasma F levels for regressional analyses; the classifications, however are based on the historical plasma F values obtained from 1987-1996.

Daily Journals and Saliva Sample Kit

In addition to their participation in the ongoing study described above, these subjects were also asked to participate in a separate study examining the relationship between cortisol, affective status and diet. Once consent was obtained, subjects were given detailed instructions regarding the methodology of the study. Following this preliminary meeting, a kit was mailed to subjects' homes that contained written instructions, sufficient filters for 30 days of saliva sampling, ziploc bags to store used filters, and numerous pairs of gloves for handling the filters. Subjects were given a booklet to record their time of sampling each day, the contents of the meals they ate, their daily activities, their emotional status at the time of sampling, and detailed accounts of any medications they took on a particular day, or any acute illness suffered during the 30 days. See Appendix for a sample of the journal.

Salivary Cortisol (30 days)

We chose to monitor cortisol levels in saliva because subjects completed this study in their homes over the course of 30 days. It has been shown that measuring cortisol in saliva provides a reliable measure of the free unbound fraction of cortisol (Kirschbaum and Hellhammer, 1994) and correlation coefficients on the order of r=0.96 between cortisol in saliva and serum have been obtained in the elderly population (Tunn et al., 1992).

Filters (3.5 x 5 cm) were cut from Whatman no. 42 filter paper (Whatman, Clifton, NJ) for the collection of saliva. The top centimetre of the 5 cm length was used for recording subject data and was the only portion of the filter that subjects were instructed to handle (to avoid any contamination). This portion was demarcated with a small cut in the filter. The subjects were asked to place the filter paper in their mouths until the saliva front reached just beyond the 4 cm line. The filter was then air-dried and stored at -4C. In previous work with this procedure, we have established that protein content, measured in 0.1-N NaOH extracts, in salivary samples collected in this manner varies by an average of less than 1% across a wide range of samples.

Cortisol was extracted from the filter in 2mL ethanol for 1 hour at room temperature. A 300 µl aliquot of the extract was assayed using [^{3}H] cortisol as radiotracer and a highly specific antibody (B-63 antibody from Endocrine Sciences, Tarzana, CA). This antibody cross-reacts less than 4% with deoxycorticosterone or deoxycortisol, and less than).5% with any other adrenal steroid. Separation of bound from unbound hormone was achieved using dextran-coated charcoal. Samples were then decanted into mini-scintillation vials, filled with 4.5 ml of Liquiscint (National Diagnostics, Sommerville, NJ) and radioactivity determined in a Packard scintillation counter at 56% efficiency. The intra- and inter-assay coefficients of variation were 3.5% and 5.0% respectively.

Over the course of 30 days, subjects were asked to provide saliva samples at four times during the day and evening: once at 8:00 am, 12:00 noon, 4:00 pm and 8:00 pm. These times were chosen to include the diurnal rhythm in secretion of cortisol. Subjects were asked to record the date and time of sampling in their diaries and on the filter papers and were not obligated to give their samples at the proposed time. They were told to use these times as a guide. Subjects stored their air-dried samples in their home freezers and at the end of the 30 days were asked to mail in their kit.

Dietary Analyses

Subjects were asked to record the contents of their meals and snacks in as much detail as possible for 30 days. This included specifying quantities, how food items were prepared, name brands of foods, when food was consumed in a restaurant (including the name of the restaurant), the time of the meal, if meals were skipped and why, and the actual description of the foods eaten. There were a number of reasons for selecting this method of collecting dietary data. First, we needed a long-term and naturalistic assessment of dietary patterns in our subjects with the least amount of intrusion as possible. Since we were interested in monitoring numerous variables at the same time (saliva F, diet and affect) over a prolonged period of time and in as natural a setting as possible, any other form of

dietary assessment, such as 24-hour recall (subjects would have to be telephoned daily and asked to recall what they ate) or food frequency questionnaires (which necessitate subjects to describe how often foods are eaten) would be intrusive and potentially disturbing and stressful. Interestingly, our elderly subjects ate very consistent types and amounts of food, which made analyzing their meals fairly straightforward. In addition, the majority of subjects provided very detailed information on the contents of their meals and the detail did not vary from one day to the next. Since we have already demonstrated individual variation in memory in this particular population, any kind of dietary recall could be affected by an inability to recall rather than differences in eating patterns. Thus the only plausible method of dietary assessment would be to have subjects record what they ate at the time that they ate it. Finally, since many subjects were retired, filling out the daily activity and dietary diary gave them a task to complete during the day and both consent and compliance were easy to obtain. McCargar et al. (1993) found very good agreement between 3-day and 7-day food records for estimating nutrient intakes and extremely high agreement between 2 consecutive three-day records. Dwyer (1994), in reviewing dietary assessment, concluded that the diet diaries are the only method for long-term at-home diet assessment and that food records were the best method for analysis of elderly eating patterns. Contents of the meals were entered into the "Foodsmart" dietary analysis program (Sasquatch software, Vancouver, BC) which breaks foods down into: total calories, carbohydrate, protein, fat, calories and dietary cholesterol, and alcohol.

Affective Status

For information on the emotional status, subjects were asked to indicate the intensity of specific emotions at their time of sampling. Every time an individual gave a saliva sample he/she was asked to record their affective status as feeling as many of the following: tired, joyful, upset, excited, depressed, energetic and stressed. Subjects rated the intensity of these feelings from "not at all, a little, medium, very much and

enormously", and for statistical purposes the rated adjectives were assigned a number with "not at all" given a 1 and "enormously" assigned a 5. The affective states rating were done 4 times a day (at each saliva sampling) for 30 days. This differed slightly from the affective status ratings used in study 2; those are featured in the appendix.

Daily Activity

Subjects were asked to describe their daily activities. This included all outings, visits to doctors, at-home activities, any illness, exercise or major event.

Statistical Analyses

The results were analyzed by repeated measures and factorial analyses of variance (ANOVA), and paired and unpaired Student's t-tests. Scheffé post-hoc tests were performed when appropriate. Correlations and regressional analysis were performed where appropriate. For the analysis of ordinal data (affective status rating scales) a Kendall-Tau non-parametric correlational analysis was used. $P<0.05$ was considered significant.

Results

Salivary F Across the Day

Salivary F levels (nmol/L) sampled at four different points in the diurnal cycle are shown in figure 60. Those subjects classified as "increasing" had significantly ($p<0.05$) elevated salivary F at all time points sampled, when compared to subjects showing stable or decreasing F levels. Subjects showed a normal circadian profile in F release, such that the levels in saliva decreased from morning into evening.

Total Calories and Macronutrient Consumption

Figure 61 shows the total average caloric intake per day. Subjects classified as "increasing" consumed a significantly ($p<0.02$) higher amount of calories per day than subjects classified as "decreasing/stable". Figures 62, 63, 64 and 65 represent the average daily fat, fat type, protein, and carbohydrate (all in grams) consumption, respectively. Individuals classified as "increasing" consumed significantly ($p<0.02$) more of each of these macronutrients when compared to those classified as "decreasing/stable". Cholesterol (mg) consumption (figure 66) was similar in both groups.

Blood Levels of Cholesterol, Triglycerides, and Glucose (final year only)

Subjects' blood total cholesterol, HDL and LDL (all in nmol/L) taken on the most recent year of the study (1996) are shown in figure 67. Subjects classified as "increasing" had higher total blood cholesterol (panel A) and LDL (panel C) and lower HDL (panel B) when compared to subjects classified as "decreasing/stable." Figure 68 is the ratio of total cholesterol to HDL(A) and the ratio of LDL to HDL (B). Individuals with increasing F had a higher ratio of total to HDL cholesterol and a higher LDL to HDL ratio when compared to individuals with decreasing or stable F. Figure 69 represents subjects' triglycerides (panel A) and blood glucose (panel B) (both in nmol/L) levels assessed on the final year of the study. Individuals classified as "increasing" had higher plasma triglyceride (panel A) and slightly higher glucose levels (panel B) when compared to individuals classified as "decreasing/stable". While subjects showing "increasing" plasma F levels with years had elevations in all the above, these were not significant.

Body Weights

Body weights (kg) taken on the final year of study are shown on figure 70. Individuals classified as "increasing" had higher body weights when compared to individuals whose F secretion is classified as "decreasing/stable."

Affective Status and Salivary F Relationship

Table 11 represents correlational analysis on time of day F levels with subjective feelings at each sampling time. Significant positive correlations ($p<0.05$ or better) were found between feeling stressed and depressed at all times of the day and salivary cortisol levels. Significant positive correlations were also found between feeling fatigued and salivary F at 8 am, noon and 8 pm. Finally, significant positive correlations were found between feelings of shock at noon and 8 pm and salivary F levels, between feeling energized and F at 8 pm, between feeling playful and salivary F at 8 am and feeling excited and F at 8 pm.

Salivary and Plasma F and Macronutrient Consumption Relationship

Table 12 shows regressional analysis between both the mean daily saliva F, time of day saliva F and the last 24-h plasma F level and various macronutrients. A significant ($p<0.05$) positive relationship was found between mean and all time of day saliva F levels (8 AM, noon, 4 PM and 8 PM) and calories, fat, protein and carbohydrate consumption, with the exception that salivary F levels at 8 PM were not significantly correlated to fat consumption. No significant relationships were found between any F levels and cholesterol or alcohol consumption.

Relationships Between Macronutrients

Table 13 summarizes the relationship between the macronutrients consumed. Significant ($p<0.05$) positive relationships between total calories and fat, carbohydrate, protein and cholesterol were found and fat, carbohydrate and protein were all significantly positively related to each other. Cholesterol was positively correlated to calories, fat and protein but not carbohydrate. Alcohol was not correlated to any of the macronutrients or total calories.

Relationships between blood levels and salivary F levels and macronutrients.

Only two significant relationships were found: a significant ($p<0.05$) negative relationship was found between HDL levels and carbohydrate consumption and a significant positive relationship was found between alcohol and HDL levels (table 14). Interestingly, HDL levels were almost uniformly negatively related to all the macronutrients and salivary F levels assessed.

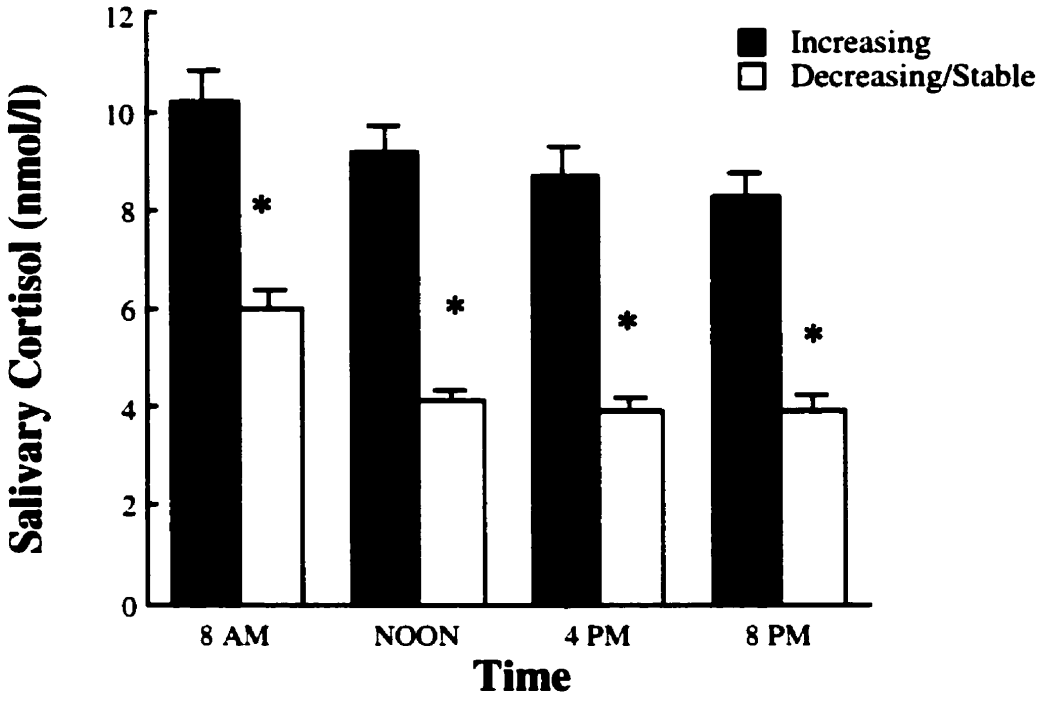

Figure 60. Mean (± SEM) average daily salivary cortisol (nmol/L) in subjects over 30 days at 8 am, noon, 4 pm and 8 pm, in elderly subjects whose cortisol secretion is classified as increasing (n=4) or stable/decreasing (n=4). * increasing significantly different from decreasing/stable.

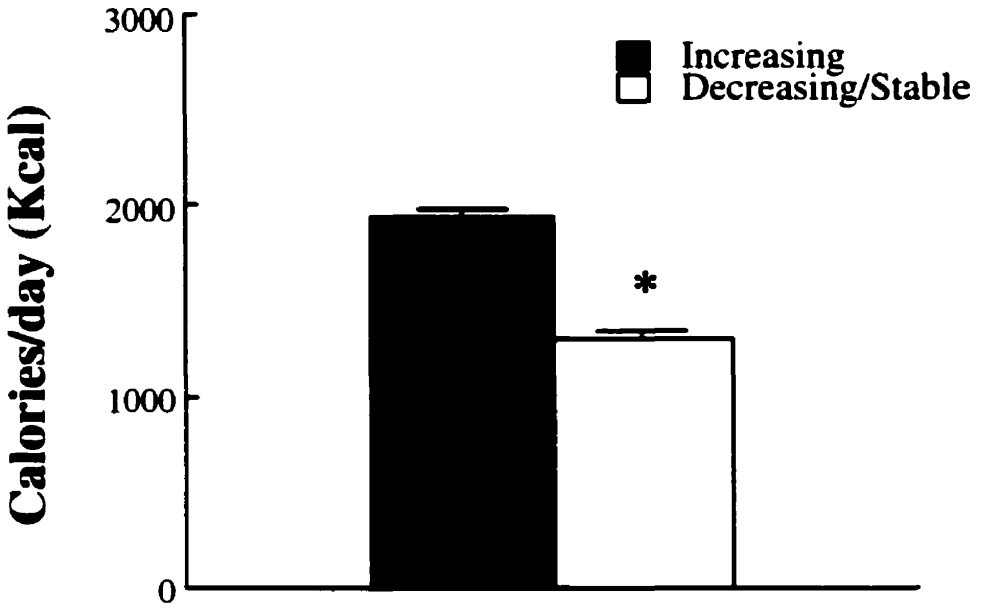

Figure 61. Mean (± SEM) average daily calories taken in over a 30 day period in elderly subjects whose cortisol secretion is classified as increasing (n=4) or stable/decreasing (n=4). * increasing significantly different from decreasing/stable.

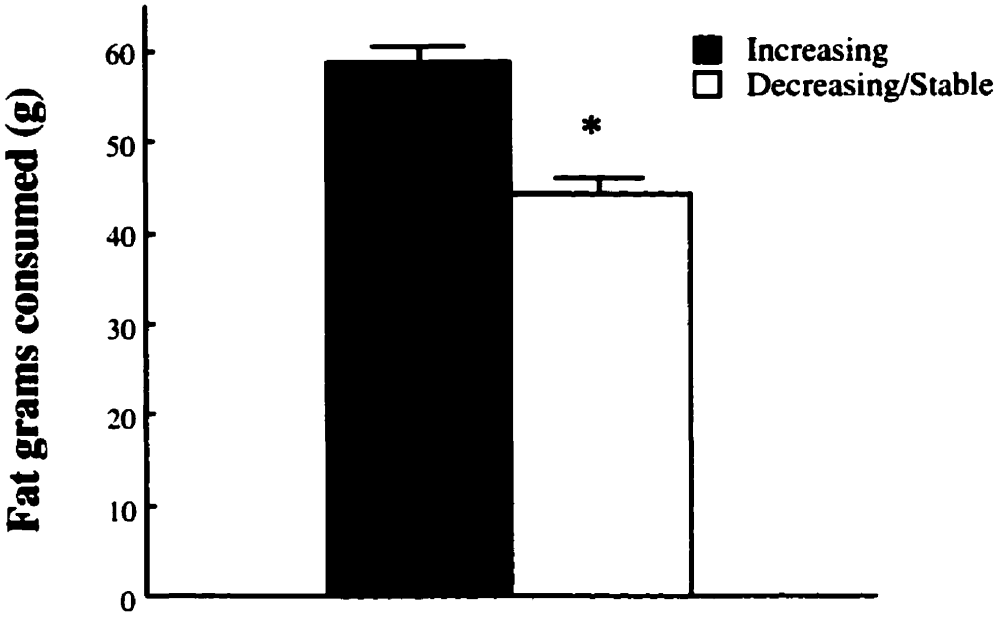

Figure 62. Mean (±SEM) average daily grams of fat taken in over a 30 day period in elderly subjects whose cortisol secretion is classified as increasing (n=4) or stable/decreasing (n=4). * increasing significantly different from decreasing/stable.

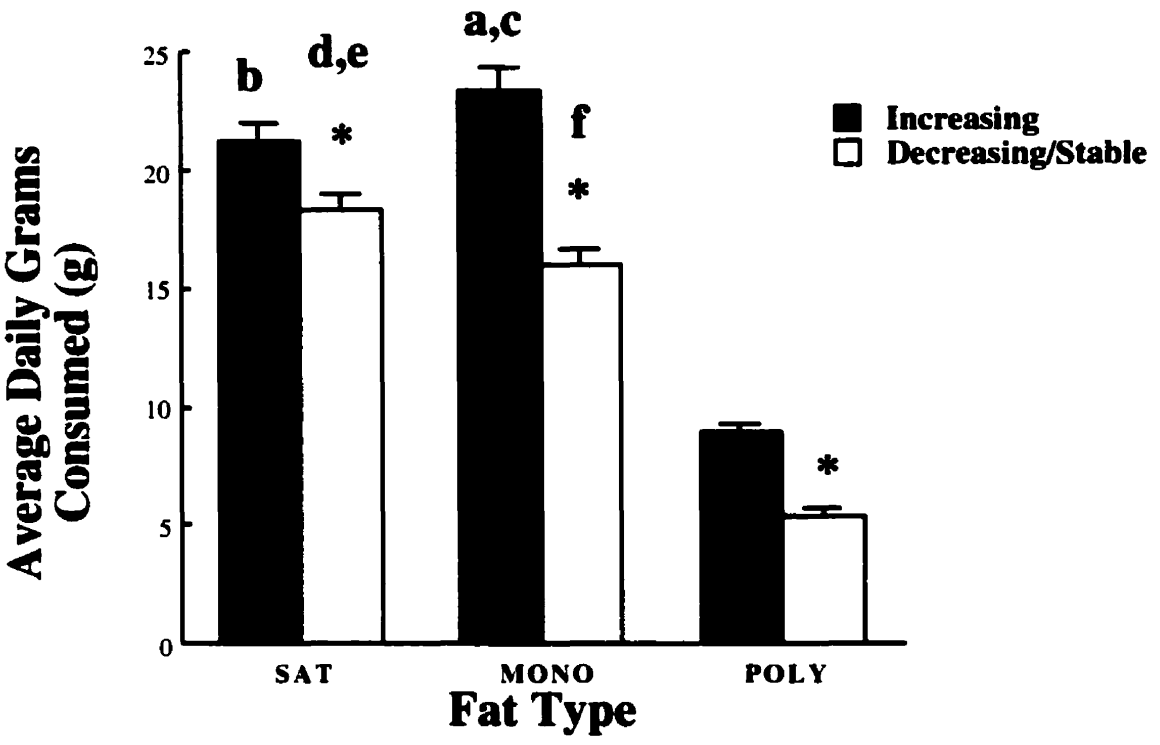

Figure 63. Mean (±SEM) average daily grams of different fat types taken in over a 30 day period in elderly subjects whose cortisol secretion is classified as increasing (n=4) or stable/decreasing (n=4). SAT= saturated fat; MONO= monounsaturated fat; POLY= polyunsaturated fat. A increasing sat different from mono; b increasing sat diff from poly; c increasing mono diff from poly; d decreasing sat diff from mono; e decreasing sat diff from poly; f decreasing mono diff from poly* increasing significantly different from decreasing/stable.

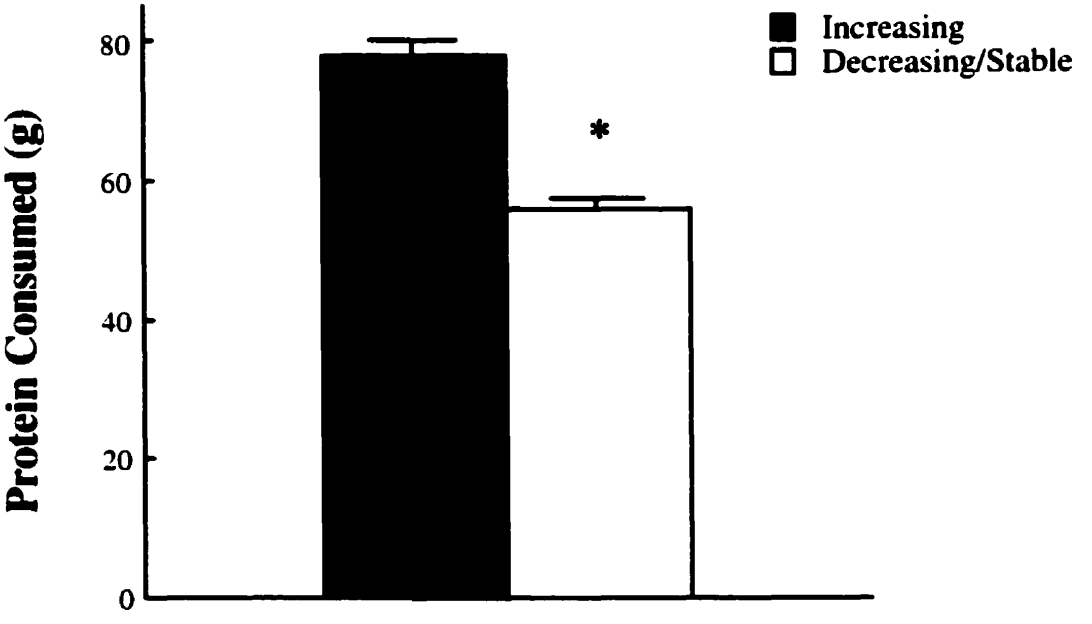

Figure 64. Mean (±SEM) average daily grams of protein taken in over a 30 day period in elderly subjects whose cortisol secretion is classified as increasing (n=4) or stable/decreasing (n=4). * increasing significantly different from decreasing/stable.

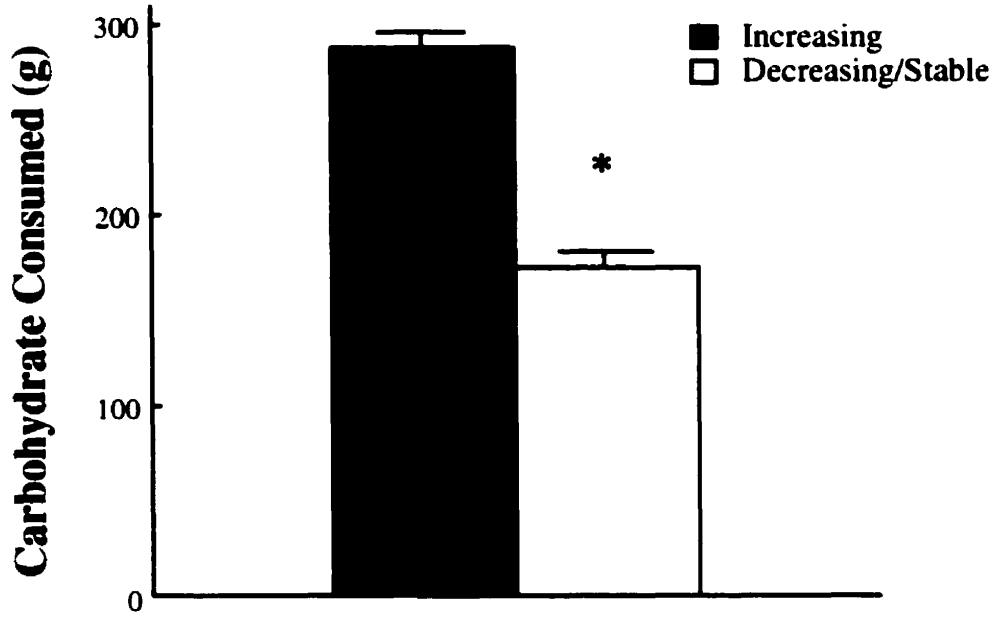

Figure 65. Mean (±SEM) average daily grams of carbohydrate taken in over a 30 day period in elderly subjects whose cortisol secretion is classified as increasing (n=4) or stable/decreasing (n=4). * increasing significantly different from decreasing/stable.

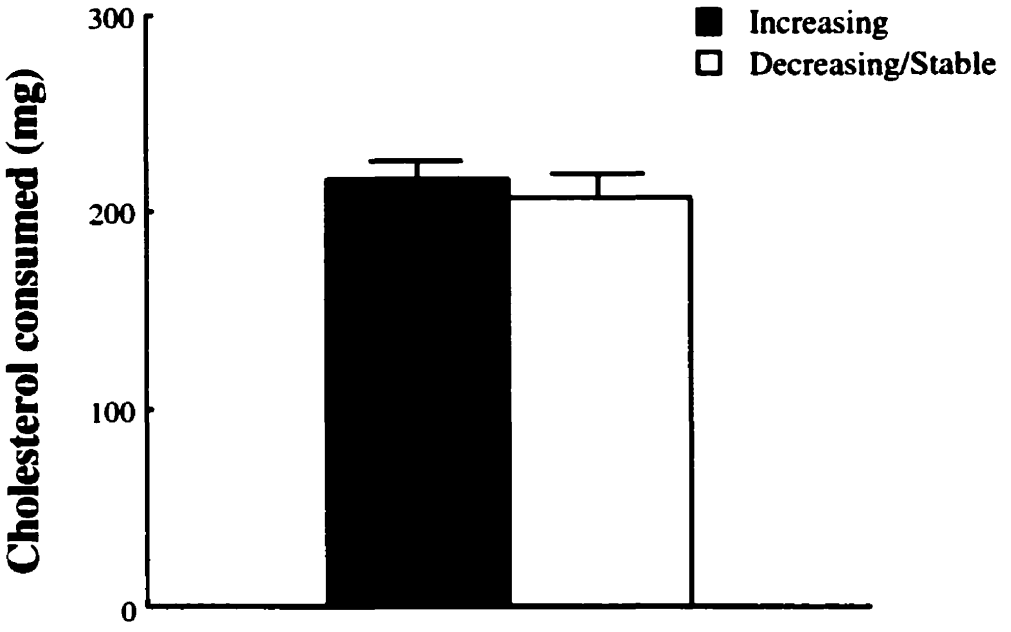

Figure 66. Mean (±SEM) average daily mg of cholesterol taken in over a 30 day period in elderly subjects whose cortisol secretion is classified as increasing (n=4) or stable/decreasing (n=4).

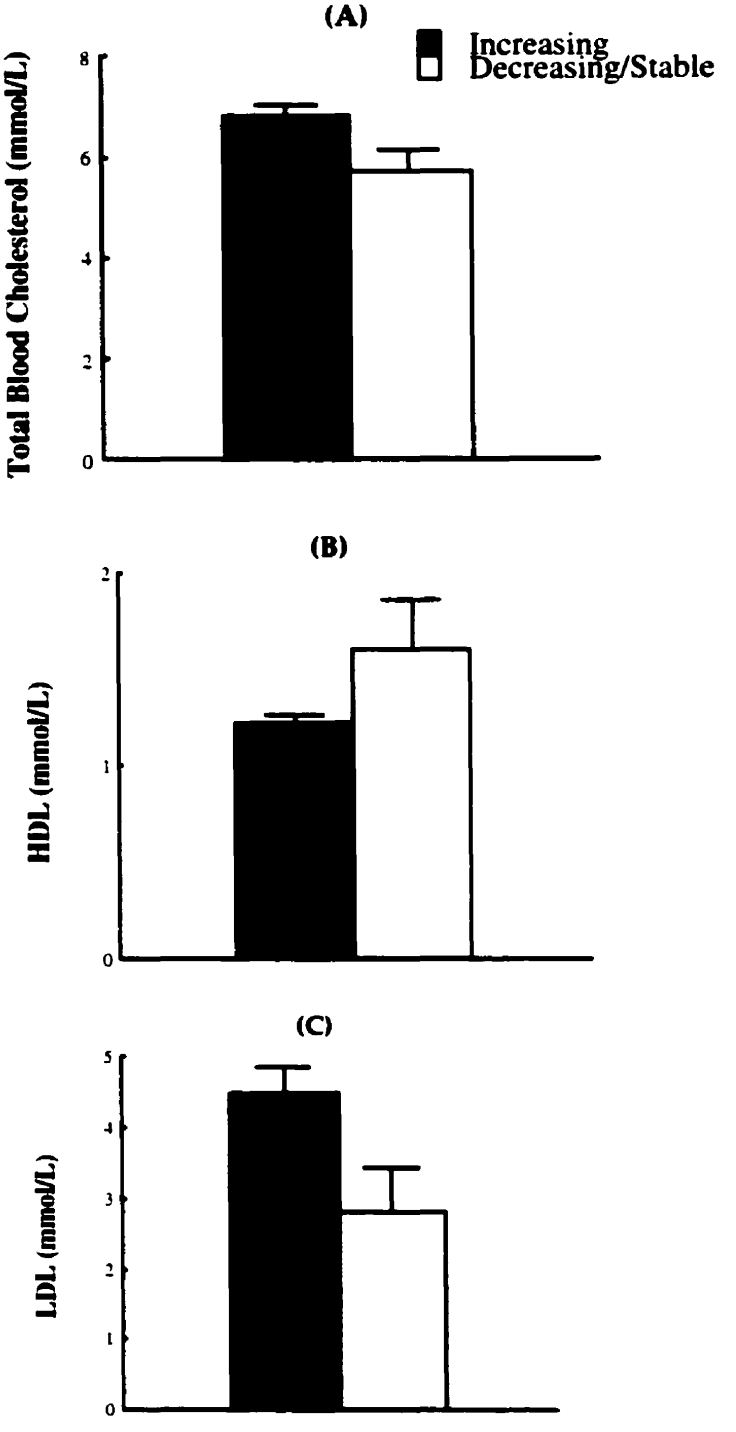

Figure 67. Mean (± SEM) total plasma cholesterol (panel A), plasma HDL cholesterol fraction (panel B), and LDL fraction (panel C) in elderly subjects whose cortisol secretion is classified as increasing (n=4) or decreasing/stable (n=4).

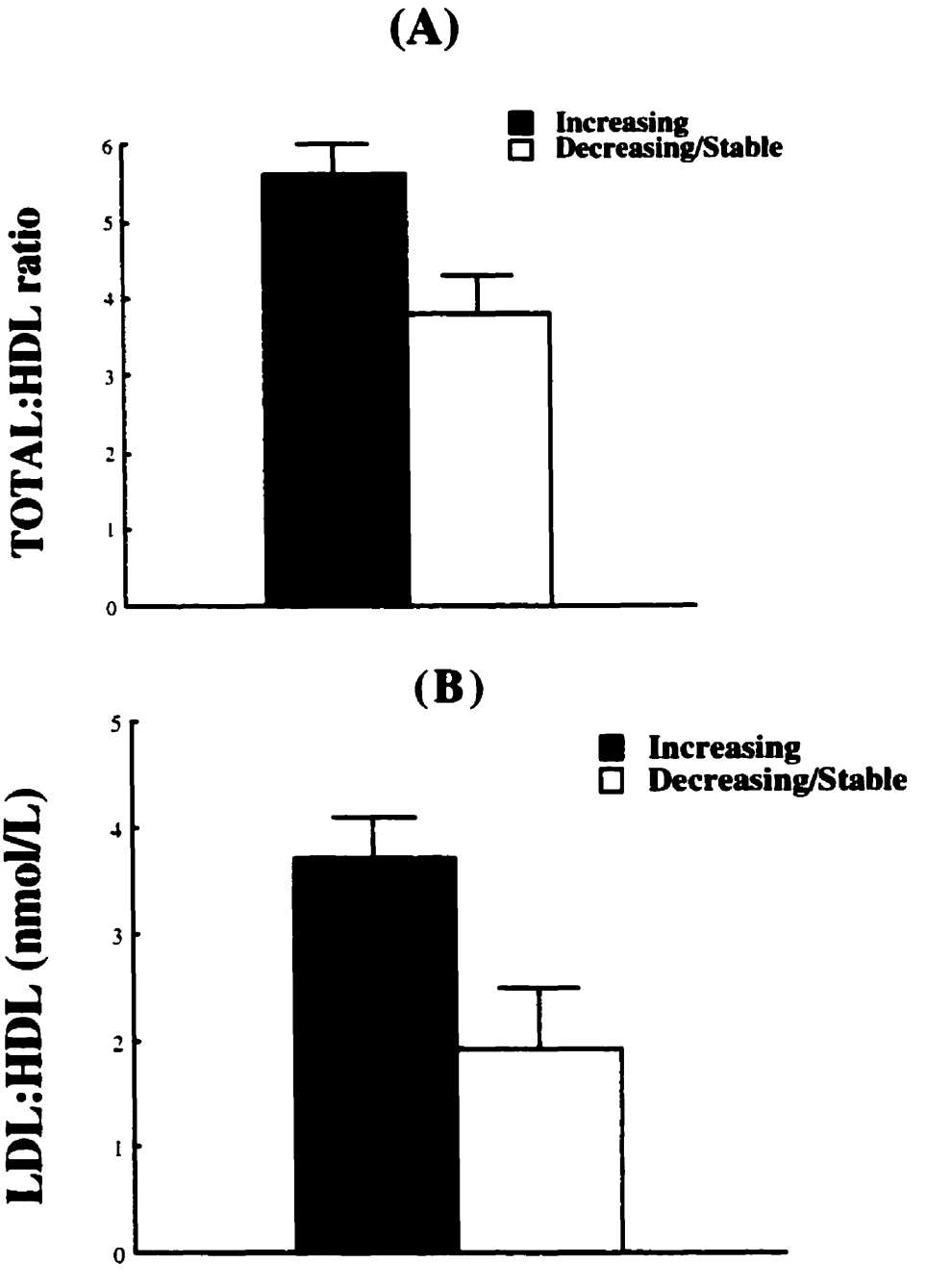

Figure 68. Mean (± SEM) total plasma cholesterol:HDL ratio (panel A), and plasma LDL:HDL cholesterol ratio (panel B), in elderly subjects whose cortisol secretion is classified as increasing (n=4) or decreasing/stable (n=4)

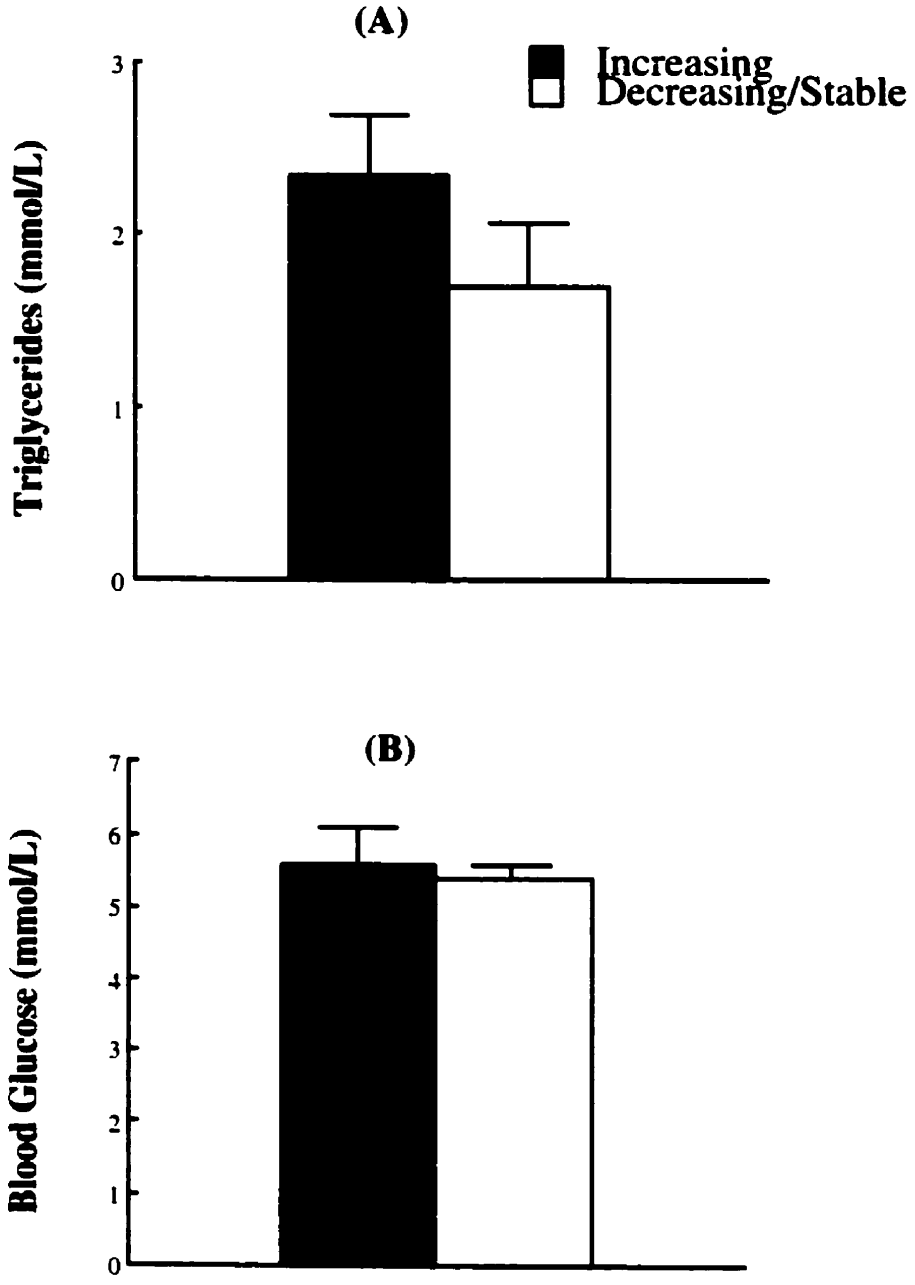

Figure 69. Mean (± SEM) plasma triglycerides (A) and blood glucose (B) in elderly subjects whose cortisol secretion is classified as increasing (n=4) or stable/decreasing (n=4).

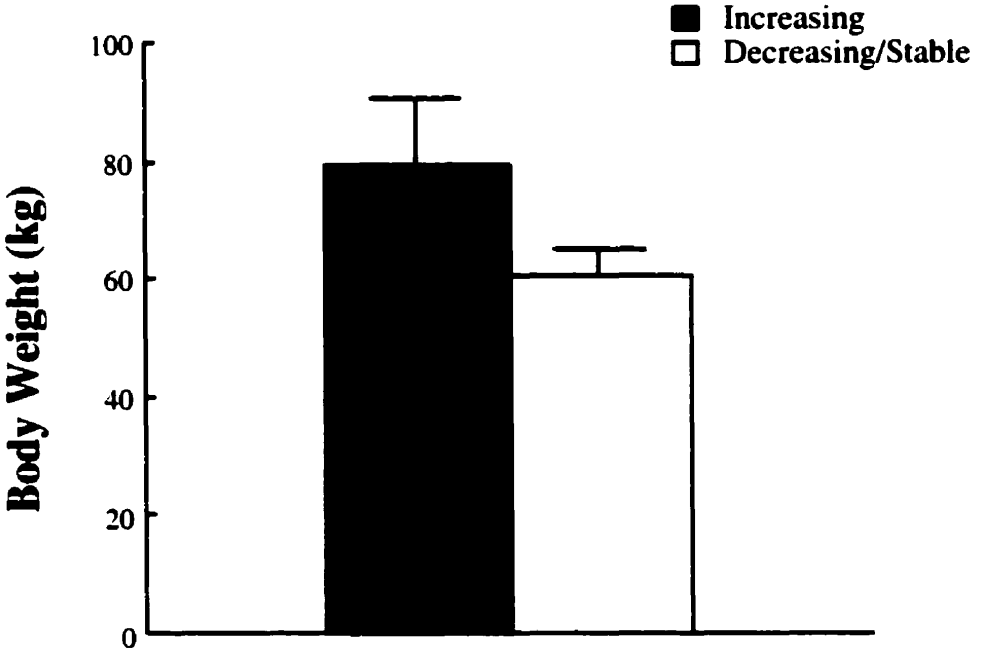

Figure 70. Mean (± SEM) body weight (kg) taken in the final year of plasma cortisol sampling in elderly subjects whose cortisol secretion is classified as increasing (n=4) or stable/decreasing (n=4).

Table 11. Kendall correlations between salivary cortisol levels at numerous times of the day and subjective feelings at the same time (n=8 subjects over 30 days/4 times per day).

	Shocked	Energized	Playful	Excited	Fatigued	Stressed	Depressed
8 AM	0.065	0.087	0.272**	0.047	0.117*	0.342**	0.246**
Noon	0.099*	0.026	-0.008	0.073	0.220*	0.376**	0.240**
4 PM	0.057	0.042	0.013	0.087	0.103*	0.304**	0.110*
8 PM	0.094*	0.108*	0.058	0.130*	0.053	0.299**	0.162**

* significant correlation at $p<0.05$

** significant correlation at $p<0.002$

Table 12. Regressional analysis between mean daily salivary cortisol levels and plasma cortisol levels and macronutrient consumption (n=8 subjects over 30 days, except plasma F was n=8 subjects averaged over 24 hours).

	Mean Daily Salivary F	8 AM F	Noon F	4 PM F	8 PM F
Calories (Kcal)	0.281*	0.226*	0.315*	0.265*	0.256*
Fat (g)	0.176*	0.134*	0.228*	0.177*	0.125
Protein (g)	0.283*	0.287*	0.272*	0.236*	0.240*
Carbohydrate (g)	0.278*	0.217*	0.305*	0.258*	0.259*
Cholesterol (mg)	0.101	0.097	0.124	0.087	0.008
Alcohol (mg)	0.025	0.027	0.029	0.015	0.024

* significant at $p<0.05$ or better

Table 13. Regressional analysis between macronutrients (n=8 subjects over 30 days).

	Calories	Fat	Protein	Carbohydrate	Cholesterol	Alcohol
Calories (Kcal)	xx	0.711*	0.702*	0.914*	0.278*	0.044
Fat (g)	0.711*	xx	0.488*	0.415*	0.456*	0.009
Protein (g)	0.702*	0.488*	xx	0.538*	0.400*	-0.042
Carbohydrate (g)	0.914*	0.415*	0.538*	xx	0.083	-0.076
Cholesterol (mg)	0.278*	0.456*	0.400*	0.083	xx	-0.044
Alcohol (mg)	0.044	0.009	-0.042	-0.076	-0.044	xx

* significant at $p<0.05$ or better

Table 14. Regressional analysis between blood levels, saliva F levels and macronutrients (n=8 subjects over 30 days, except blood levels were assessed once or averaged over 24-h for cortisol).

	Total blood cholesterol	HDL	LDL	Triglycerides	Blood Glucose	Body weight
Calories (Kcal)	0.153	-0.651	0.557	0.297	0.118	0.115
Fat (g)	0.221	-0.447	0.300	0.027	0.513	0.247
Protein (g)	0.570	-0.295	0.594	0.307	0.135	0.222
Carbohydrate (g)	0.017	-0.743*	0.536	0.346	0.074	0.035
Cholesterol (mg)	0.141	0.101	0.472	0.563	0.406	0.125
Alcohol (mg)	0.024	0.745*	-0.234	-0.236	-0.300	-0.351
8 AM saliva F	0.514	-0.168	0.178	0.572	0.094	-0.173
Noon saliva F	0.519	-0.315	0.396	0.607	0.188	-0.102
4 PM saliva F	0.524	-0.305	0.422	0.594	0.099	-0.106
8 PM saliva F	0.572	-0.174	0.504	0.535	0.012	-0.129
Mean daily saliva F	0.555	-0.216	0.377	0.586	0.091	-0.136
Last 24-h plasma F	0.341	-0.081	0.119	0.373	0.084	0.333

* significant at $p<0.05$ or better

Discussion

Individuals who has been classified as those with increasing plasma F levels over years and also elevated plasma F in the latest year of the study also have elevated salivary F levels at all points in the cycle when compared to individuals showing stable current plasma F levels or stable or decreasing plasma F with increasing years. This is important because it confirms that the assessment of F levels in saliva is an accurate representation of plasma F levels. Accurate assessments from saliva allow for a non-invasive measure of the dynamic change in F levels over a longer period of time (30 days), as opposed to the one-day method which may only reflect one static period. In addition, the non-invasive at-home sampling accurately reflects naturally fluctuating F levels, as opposed to the laboratory environment, which may artificially elevate plasma F. Levels were fairly stable within each individual over time (note the small amount of variation) which further validates the method and confirms the consistency of these levels over time. We also found that individuals who has been characterized as "increasing" with respect to their plasma F levels showed a smaller decrease in salivary F from 8 AM to noon when compared to individuals classified as "decreasing/stable." This may suggest that levels in saliva were chronically elevated (there is also very little decrease from noon to 4 PM and 4 PM to 8 PM) throughout the entire diurnal cycle.

It is not surprising that those individuals with the increasing plasma F and elevated salivary F consume the most calories. A substantial body of evidence suggests a role for F (in humans) and B (in rats) in caloric storage and in the control of food consumption. A number of critical studies have shown that GCs exhibit a dose-response curve on their effects on nutrient ingestion and metabolism, with their anabolic actions predominating at low-to-moderate levels (Tempel and Leibowitz, 1994). Cohn et al. (1955) was one of the first to demonstrate that adrenalectomy (ADX) decreases food intake and weight gain compared to rats with intact adrenals. In adx-ed rats, 24-hour caloric intake and body weight gain can be restored by low to medium chronic doses of B, which raise blood levels

to 1-10 μg% (Kumar and Leibowitz, 1988; Dallman et al., 1989) and restore the consumption of both carbohydrate and fat and the deposition of fat (Steele, 1975;. Tempel et al., 1991).

The finding of a reduction in caloric intake in individuals with lower salivary and plasma F levels raises another interesting issue. There is a growing body of evidence that links lifelong caloric restriction with a slowing or even retardation of brain and body aging as well as increasing maximum lifespan (Ingram et al., 1987; Weindruch and Walford., 1988). A number of groups have found that caloric restriction before or at mid-life prevents certain learning deficits seen in aged rats that have not been calorically restricted (Ingram et al., 1987; Idrobo et al., 1987; Means et al., 1993; Dubey et al., 1996). Prolonged caloric restriction can also affect the integrity of those brain areas that subserve certain cognitive functions, such as the hippocampus. Under the usual conditions of caloric restriction experiments, significant increases in GC levels only occur during the early stages of restricted feeding (Stewart et al., 1988; Sabatino et al., 1991) suggesting that hypercorticism is not a necessary consequence of caloric restriction. In addition, it is perhaps the concurrent F levels that are most important, i.e. individuals who show cognitive integrity have current cortisol levels that are in the normal range. Further our subjects are free feeding such that their consumption is not so much restricted as it is reduced. The hippocampus is highly sensitive to caloric restriction and to circulating GC levels, such that chronic elevations in B or F can accelerate hippocampal aging, which in turn may be responsible for cognitive impairments; caloric restriction in our subjects does not result in chronic elevations in F. Hori et al. (1992) found that LTP was preserved in aged mice that had been calorically restricted since weaning; aged mice that had received ad lib calories throughout life showed a reduced PTB and LTP compared to those calorically restricted. More recent work has demonstrated that caloric restriction may relieve age-associated levels of oxidative stress (via a reduction in protein damage to hippocampal neurons) and may also cause an upregulation of synapsin phosphorylation, which is often reduced in aged rats (Eckles et al., 1997).

This present study and Lupien et al's (1998) work extends these findings to the human: individuals who consume fewer calories on average, are the same individuals with reduced plasma and salivary F levels. They also perform better on cognitive tasks known to be hippocampal sensitive and have a significantly greater hippocampal volume compared to individuals who consume a greater amount of calories (and are those individuals with higher plasma and salivary F (Lupien et al., 1998). Thus, one potential source of variation between individuals with increased F and concomitant cognitive impairment and those whose levels remain stable over time and who do not show cognitive impairment is the overall number of calories they take in.

We found very strong positive relationships between mean daily saliva F levels, saliva F levels at each time point sampled and all the macronutrients examined, with the exception of dietary cholesterol. This relationship has been examined in numerous animal studies. In adx-ed rats, the daily ingestion of fat is decreased by approximately 30%, lending support for the involvement of glucocorticoids in the consumption of fat. Increased F production is related to increased preference for and intake of fat, which has been observed in obese humans (Horber et al., 1986; Castonguay, 1991). Peripheral administration of RU28362 as well as B reveals a strong stimulatory effect on carbohydrate intake in rats (Tempel et al., 1992); this is abolished by RU2832, a GR antagonist and by adrenalectomy as well. We found very strong associations between carbohydrate consumption and F levels at all times of the day while fat consumption was not as strongly associated. This confirms animal studies that suggest that low to moderate levels of B seem to potentiate fat consumption while higher B potentiates carbohydrate consumption. Protein consumption was also higher in individuals with elevated salivary F. Bligh et al. (1993) have shown that ADX reduces protein consumption and that B supplementation in ADX rats restores protein intake (Kumar et al., 1988). We also examined the relationship between the macronutrients themselves to determine if consumption of one was partly dependent on the consumption of another. We found very strong and significant relationships between the

consumption of total calories and all the macronutrients (see table 3) with the exception of alcohol. This simply confirms that our subjects' total caloric intake is made up of fats, proteins and carbohydrates and not one particular macronutrient.

Macronutrients themselves have direct effects on circulating B. High-fat feeding elevates both basal and stress-induced HPA function. Carroll and Noble (1952) first demonstrated that feeding rape oil to rats caused an increase in stimulated B levels in rats, as early as after 3 weeks of feeding. Hulsmann (1978) and Brindley et al. (1981) reported similar findings. Pascoe et al. (1991) found that 3 weeks of feeding rats a high-fat diet caused an increased B and glucose response to a swim stress. Tannenbaum et al. (1997) found that basal B, glucose and free fatty acid (FFA) levels were affected by as little as 7 days of high-fat feeding; ACTH and B responses were stress were also elevated. Widmaier et al. (1992) demonstrated that direct FFA infusion via the jugular vein increased plasma B in rats for up to 2 hours following the infusion. Finally, Kamara et al.,. (1998) showed that rats fed high-fat diets show an increased and prolonged plasma B response to an acute stress. In rats within a normal range of adiposity, high levels of B are associated with a high carbohydrate and high protein diets and lower body weight (Wang et al., 1998). This is consistent with other reports in rats (Boivin and Deshaies, 1995) and humans (Gardner and Reiser, 1982).

When we assessed the relationship between macronutrient consumption and plasma levels of blood cholesterol, HDL, LDL triglycerides, glucose and body weight, we only found a significant negative relationship between HDL levels and carbohydrate consumption. Excessive carbohydrate consumption has been associated with ischemic heart disease and may elevate serum lipid levels (Schonfeld, 1985). Dietary carbohydrates have been shown to reduce the amount of HDL in the serum (Anderson and Seiling, 1985); this kind of relationship was found in our subjects as well. Although we did not see a significant elevation in triglycerides in subjects who consumed more carbohydrates, their

significantly decreased HDL levels may indicate a first step in the pathogenesis of coronary artery disease (CAD). Another possibility for a lack of a relationship between macronutrients and plasma indicators of CAD and diabetes is the dynamic fluctuation in blood levels might not have allowed for an accurate assessment since blood samples are drawn once a year. Most clinical recommendations include multiple samples to accurately assess risk factors for CAD and diabetes.

There is also a significant relationship between obesity, increased abdominal body fat deposition and F levels in humans. Hypercortisolism favors fat accumulation in visceral depots, causing android obesity and weight gain. GC supplementation induces weight gain and adiposity in humans (Horber et al., 1986). In Cushing's syndrome, chronically elevated levels of GCs, frequently similar to those seen in non-Cushing's patients with visceral obesity, are associated with the pathophysiology that is seen in the metabolic syndrome, namely polyphagia, weight gain, marked visceral obesity, fatigue, muscle wasting, anergia and atypical depression (Peeke and Chrousos, 1995). Rosmond et al. (1998) found that a increases in saliva F over the diurnal cycle are associated with problems in the homeostasis of several somatic systems. They found significant positive correlations between F levels and obesity factors, such as waist-hip ratio, insulin, glucose, triglycerides, total and LDL cholesterol. A number of studies in primates suggest that when cynomolgus macaques are exposed to chronic social stress, they show evidence of the metabolic syndrome: high visceral fat deposition, incidence of coronary artery atherosclerosis, hyperglycemia and insulin resistance, higher blood pressure, adrenal gland hypertrophy, enhanced adrenal responsiveness to ACTH, and higher total cholesterol and lower LDL concentrations (Shively et al., 1987). Socially subordinate baboons had significantly decreased HDL and elevated cortisol levels (Sapolsky and Mott, 1987) and both acute and repeated stressor exposures causes increased plasma cholesterol in rats (Berger et al., 1980; Servatius et al., 1993; 1994) and humans (Dimsdale and Herd, 1982; McCann et al., 1990).

In the assessment of the relationship between salivary F and affective status, we found significant positive relationships between cortisol levels at all times of the day and feelings of stress and depression. A large body of literature suggests a hyperactive HPA axis in depressed patients (Arborelius et al., 1999). Board et al. (1956) was one of the first to report elevated plasma F concentrations in a majority of patients with major depressive disorder; this finding has been replicated repeatedly. Individuals who report feelings of chronic stress and depression also exhibit elevations in both plasma and urinary F levels, suggesting a tight relationship between the perception of stress and the reactivity to it (Ravindran et al., 1999). Interestingly, we also found that individuals with higher F levels also rate themselves as feeling excited at 8 PM and then playful at 8 AM. This is critical for two reasons: there is some evidence for a role for corticosteroids in increasing feelings of elation and hyperactivity (Starkman et al., 1981). Also this kind of mood rating may have been a semi-conscious attempt at coping with feelings of depression and stress and was therefore reported when negative affective states were reported at the same time.

A number of mechanisms for GC-controlled intake of total calories, fat, protein and carbohydrate have been investigated. B levels may affect nutrient stores through its permissive interactions with specific hypothalamic neurochemical systems. Both neuropeptide Y (NPY) and noradrenaline (NE) strongly potentiate food intake and cause a preferential enhancement of carbohydrate ingestion (Stanley et al., 1985; Leibowitz and Alexander, 1991). These neurochemicals also have a stimulatory effect on B release (Leibowitz et al., 1988). The feeding-stimulatory action of NE, via alpha-2 receptors in the PVN, is dependent on and positively correlated with circulating B. Therefore, one possibility is subjects with elevations in plasma and salivary F also have elevated NE levels, which are responsible for both an increase in carbohydrate consumption and in circulating F. Interestingly, a bidirectional relationship might exist between NPY and carbohydrate consumption: the amount of carbohydrate consumed under natural feeding conditions is positively correlated with NPY levels in the arcuate and paraventricular nucleus (ARC and

PVN respectively) (Jhanwar-Uniyal et al., 1993; Wang et al., 1998a). In rats given a single high carbohydrate diet, higher NPY gene expression was found in the ARC compared to animals fed a high-fat diet (Giraudo et al., 1994; Wang et al., 1998a). A more recent report by Wang et al.(1998c) suggests that animals who consume a high-carbohydrate diet have higher levels of circulating B when compared to rats who consume diets that are lower in carbohydrate. Elevations in circulating plasma F following the prolonged consumption of carbohydrates has also been found in humans (Gardner and Reiser, 1982).

Individuals with higher body weights also had higher salivary and plasma F. A number of groups have shown that repeated hypothalamic injections of NPY enhance body weight (Stanley et al.,1986). At the same time, NPY and B raise circulating levels of insulin and in time, to the point of insulin insensitivity (Dallman et al., 1993); this change ultimately results in an accumulation of body fat (Zarjevski et al., 1993). Therefore this pattern of high insulin, B or F, NPY and carbohydrate intake reflects an anabolic state that promotes a shift in energy stores towards lipid formation. Another neuropeptide, galanin (GAL), stimulates fat and carbohydrate consumption when injected into the PVN (Wang et al., 1998b; Leibowitz and Kim., 1992; Tempel and Leibowitz, 1993). In fact, GAL stimulates the ingestion of fat and fat consumption, enhances the activity of GAL (Leibowitz et al., 1998; Wang et al., 1998b).

In addition to the effects of NPY and galanin on GC-controlled metabolism, the antagonistic relationship between insulin and GCs should be considered as well. In the absence of insulin, as in the diabetic rat, or in states of insulin insensitivity, increases in chronic plasma B levels to the stress range cause continued increases in food intake (Dallman et al., 1993), compared with a smaller, saturable increase in plasma B in non-diabetic control rats. This may be a consequence of the lack of a postulated inhibitory effect of insulin on food intake. While insulin was not measured in our subjects, basal glucose levels in individuals with increasing F and increased caloric consumption and those

with lower F and caloric consumption were similar. However one cannot rule out the possibility for the emergence of glucose intolerance in our subjects with increased plasma and salivary F; despite a normal range of basal fasting plasma glucose, carbohydrate metabolism may nevertheless be abnormal as reflected by the plasma glucose response to an oral or IV glucose load (Felig et al., 1995). Since the response to a glucose load was not measured in our subjects, it is impossible to rule out this possibility. Considering that GC antagonize many of insulin's effects, it would seem plausible that glucose control in individuals with elevated plasma and salivary GCs (perhaps through an increased consumption of fats and calories) gets shifted to be under the control of GCs. An increased control of metabolism by GCs is characteristic of many of the risk factors for atherosclerosis (Brindley and Rolland, 1989). Interestingly, subjects with elevated GCs also have elevations in triglycerides, total blood cholesterol and LDL, and decreased HDL, all biomarkers for the development of CAD. In fact, we have previously reported that individuals with increasing F levels with years presented with a significant increase of triglyceride levels with years when compared to the other two groups (Lupien et al., 1995). When cholesterol ratios were assessed, individuals with higher F also had higher ratios of both total to HDL and LDL to HDL cholesterol. This is a critical finding in that "increasing F" individuals had cholesterol ratios in the high-risk/dangerous range, in accordance with numerous studies (Masuoka et a., 1998) while "decreasing/stable F" individuals were in the target range for desirable levels; these ratios are considered as better markers for risk factors for the development of CAD. Again, these data show the importance of F levels as a biomarker for the development and progression of disease, as GCS can directly affect both HDL levels as well as the receptor for LDL, exaggerating conditions such as hypertriglyceridemia (Brindley and Rolland, 1989).

Taken together, these results suggest that one potential source for the variation in plasma and salivary F levels is individual variation in food consumption. While life-long dietary habits may themselves have a permanent impact on the risk for the development of

metabolic and cognitive pathology, their effect on chronic cortisol levels may be a potential window into how diet-induced changed in GCs may themselves impact the emergence of biomarkers associated with disease and the process of aging. The possibility exists for the reverse relationship: potentially beneficial interventions introduced at mid-life, known to affect systems that are associated with HPA axis functioning, might prevent some of the age-associated pathology associated with chronic life-long exposure to the highly catabolic GCs.

Study 6 The Effects of Environmental Enrichment on HPA and Metabolic Function in Aged Rats

The effects of manipulating the housing and rearing environment of rats have been studied extensively. Hebb (1949) was one of the first to report that animals reared in a "free environment" acquired the strategy to solve a complex problem-solving tasks faster than rats from a "restricted environment." These were followed by studies by Rosenzweig (1979) who set up the enriched versus impoverished environmental paradigms. Environmentally enriched (EE) rats are housed in large cages that contain ladders, toys and tunnels changed on a weekly basis. Juvenile and young adult rats housed in enriched environments undergo a series of neurochemical, neuroanatomical and behavioral changes. The behavioral changes that EE rats display include lower emotionality scores and greater exploration time in the open field when compared to rats that have not been enriched or have been socially isolated. (Fernandez-Treul et al., 1997), and improved performance on the Morris Water Maze (Mohammed et al., 1990; 1993; Henriksson et al., 1992). Environmental enrichment lowers anxiety in the presence of a predator stress; enriched rats spend more time in close proximity to a cat (Klein et al., 1994). Haemisch et al. (1994) showed that mice in the enriched condition attacked intruders significantly more frequently than mice that were group housed in standard laboratory cages. Conversely, environmentally impoverished (EI) rats housed individually in single, opaque cages show

behavioral and physiological alterations including deficits on the acquisition of spatial tasks, increased startle responses (Domeney and Feldon, 1998) and alterations in HPA activity (Gamallo et al., 1986: Mar Sanchez et al., 1998).

Some of the neurochemical and neuroanatomical changes that can occur in response to both brief and longer periods of EE include increased dendritic branching, increased number of synapses per neuron and synaptic size (Turner and Greenough.,1983; Volkmar and Greenough 1972; West and Greenough., 1972) primarily in the hippocampus, visual cortex, and cerebellum. More recent work in this field has demonstrated a link between increased hippocampal nerve growth factor (NGF) levels Olsson's group (1994) has shown that environmental enrichment induces glucocorticoid receptor (GR) expression in specific hippocampal subfields (CA1 and CA2) and an increased expression of mRNA encoding the NGF-induced immediate early gene NGFI-A in CA2. In addition to the effects of EE on anatomical brain plasticity, a number of studies have focused on how other systems may be affected by this manipulation. Park et al (1992) has shown that enrichment causes an increase in hippocampal acetylcholine (ACh) synthesis. It is well known that the cholinergic system plays a critical role in learning and memory (Brito et al., 1983).

A number of studies have examined the effects of EE on the aging process, in terms of synaptic plasticity, endocrine profiles and behavioral parameters. EE modifies the sleep-wake pattern of aged rats, by increasing the amount of time spent in slow wave sleep (SWS) and desynchronized sleep, which is what is generally found in younger rats (Mirmiran et al., 1986; Van Gool and Mirmiran, 1986). Similarly, the learning performance in aged rats that had spent a year in EE was improved when they were tested at 2 years of age (Doty, 1972). Rats housed in EE conditions for 509 days had higher brain weights, longer cerebral length and made significantly fewer errors in the Hebb-Williams maze than those reared in isolation (Cummins et al., 1973). Aged rats that were 766 days old and exposed to EE conditions until they were 904 days old had significantly

thicker cerebral cortices (Diamond et al., 1985). Greenough's group (1986) has shown that EE can cause significant changes in the dendritic field of the cerebellum of EE-housed aged rats. Escorihuela et al. (1995) investigated the effects of a 6 month period of EE (which began 3 weeks after weaning) on spatial learning in 24-month old hypo- and hyper-emotional and rats. EE during adolescence and early adulthood prevented later age-associated learning deficits in both rat lines. These studies suggest that the effects of EE are permanent and may persist into old age in numerous rat strains.

While the effects of EE on many behavioral and neuroanatomical systems have been examined, the mechanisms underlying the effects of EE remain unclear. To date, very few studies have described basal and stress-induced glucocorticoid status following brief or prolonged periods of EE. Basal HPA activity tends to increase with age in the rat, reflected in elevated plasma levels of ACTH and B (Sapolsky et al., 1983; Sapolsky, 1991; Meaney et al., 1988; 1991; 1992). This increase in HPA activity appears to occur as a function of dampened glucocorticoid (GC) negative feedback inhibition over HPA activity and is associated with a loss of glucocorticoid receptors in the hippocampus (Sapolsky et al., 1983; Ritger et al, 1984; Meaney et al., 1988; 1991; 1992), a crucial site for feedback inhibition. Increased basal and stress levels of B are associated with a loss of neurons in the hippocampus, an area that is critical for spatial learning. Issa et al. (1990) found that only those aged rats selected for spatial impairments showed a concomitant increase in both basal and stress-induced HPA activity, a substantial loss of hippocampal neurons and GR, and impaired acquisition of the Morris Water Maze. The hippocampus has been thought of as a primary target of plasticity in response to changes in the environment. Therefore if EE can induce changes in hippocampal GR, NGF-IA levels or other indices of plasticity that are sensitive to circulating B levels, then it follows that perhaps modifications in those systems following EE might have an effect on lowering B levels. A reduction in basal B, either as a direct result of EE or due to changes in hippocampal morphology in response to

EE, may then attenuate hippocampal aging and prevent spatial memory deficits from emerging during aging.

In addition to changes in HPA function, carbohydrate metabolism (glucose tolerance and insulin sensitivity) can also change with age. In particular, circulating glucose levels have been shown to increase with age, although the emergence of glucose profiles in aged animals and humans seems to be more dependent on lifestyle factors such as diet and physical activity (Rowe and Khan, 1987). Many experiments have studied the memory-improving action of glucose in both aged and young animals (Gold, 1995; Messier and Gagnon, 1996; White, 1991; see previous chapter and introduction). Glucose has been found to improve encoding and in some cases, retrieval, in a variety of tasks (Messier and Gagnon, 1996) and can attenuate novelty-induced anxiety (Kopf and Barrati. 1996). A number of mechanisms for the glucose potentiation of hippocampal-dependent learning include enhanced cholinergic function, which also occurs in response to EE (Korol and Gold, 1998). Of particular importance is the finding that exogenous glucose administration seems to enhance memory function, particularly in aged rats and under conditions of active learning (Korol and Gold. 1998). Therefore if one considers EE as consistently stimulating, and EE is associated with a sparing of cognitive deficits, particularly in those tasks that are subserved by the hippocampus, then perhaps EE preserves hippocampal function via its effects on both glucoregulatory mechanisms and HPA function. In addition the HPA and glucose-insulin axes are both players in controlling blood glucose levels, and hence environmental manipulations may affect the interplay between the two.

We therefore examined changes in basal and stress-induced HPA functioning in response to prolonged periods of EE imposed at mid-life. To get at the question of the effects of the environmental enrichment itself (as opposed to the effects of the social setup) we had an additional group that was a purely social (no access to toys). In addition we

included a socially isolated group as a control for the social aspect of the study. We also had an aged control group to examine the effect of age on a number of our variables. The behavioral battery included tasks that are known to be glucocorticoid, hippocampal- and age-sensitive, namely acoustic startle, morris water maze and open field testing. Finally, since aging is also associated with changes in glucose tolerance, we decided to explore indices of carbohydrate metabolism.

Methods

Animals

Long-Evans male rats (Charles River, St. Constant, Quebec), aged 12 months, were used in the study. A young control group of 6 month-old rats, were used for testing at the end of the study only; these animals were received at 3 months of age weighing between 250-350 grams and were housed in standard laboratory cages for 3 months prior to testing (2 per cage). Twelve month old animals weighed between 750-850 grams at the onset of the study. Rats were housed on a 12:12 light-dark cycle (lights on at 0800) and were housed 2 per cage until the beginning of the study. Prior to the beginning of the study, rats were housed in a humidity- and temperature-controlled environment with ad lib access to food (Purina Lab Chow) and water. Animals were randomly assigned to one of four groups: a) Enriched animals (n=15) were housed 3 per cage in large, spacious cages and given a choice of toys to manipulate (balls, bells, tunnels, ladders, chewing toys). These toys were changed at least once per week. Each rat had approximately 125.42 cm^2 of floor space. b) Socially-grouped animals (n=15) were group housed in a multi-leveled, spacious apparatus (built by Precimax Inc., Lasalle, Quebec) that was connected by tunnels. The placement of food and water was routinely changed so that animals would have to move from one level to the next in order to eat and drink. In this condition, each rat had approximately 182.76 cm^2 of floor space. c) Isolated animals (n=15) were housed in

standard, opaque laboratory cages in order to restrict visual and partial olfactory cues from other rats. d) Control animals (n=15) were housed 3 per cage and had approximately 125.42 cm^2 of floor space (identical to the enriched rats), but had no access to toys. All of these housing conditions lasted for 12 months. Health of the animals was monitored regularly by a veterinarian and animals found with any health problems (respiratory difficulties, tumours) were eliminated from the study. Housing and experimental procedures were conducted according to the Canadian Council on Animal Care guidelines and were approved by both the Douglas Hospital and McGill University Animal Care Committees.

Procedures

Measures taken at the onset and throughout the study

In order to assess changes in basal plasma B in response to changes in the housing environment, blood was sampled from the tail vein at three points throughout the study: at the onset, prior to assigning animals to housing conditions ("B1"), 7 months following the onset of the varied housing conditions ("B2") and at the termination of the study ("B3"). These samples were taken immediately following lights on (AM at 0800h) and off (PM at 2000h) to get a measure of the diurnal variation of plasma B. Blood was sampled from the tail vein (300 µl of blood) and sampling was completed in less than one minute following removal from the home cage (Viau et al., 1993). Blood samples for B measurement were collected into tubes coated with EDTA placed on ice, and then centrifuged and stored at -20°C until assayed. Body weights and food intake were monitored throughout the study. Body weights were taken approximately every two to three weeks. Food intake was measured every day over a period of 3 weeks (4 months after the introduction of the different housing manipulations) by subtracting uneaten food plus spillage from total food provided; spillage was collected on a diaper placed under the cage. Data on food intake

was analyzed per rat: the mean of intake per cage was divided by the number of rats in that cage. Then the mean for animals in a particular group was calculated and represented.

Measures taken at the termination of the manipulations imposed

After 12 months exposure to one of the four environments, rats were assessed on a number of behavioral, endocrine and metabolic measures.

a) Open Field Testing

Animals from all groups were placed, one at a time, in a novel, circular open field, 1.6 m in diameter. Animals were allowed to explore for 10 minutes. The critical measures were: time spent exploring both the inner and outer area of the novel open field, the latency to emerge into the inner portion of the open field, and the time spent frozen. Exploration was defined as the entire body of the animal being away from the immediate vicinity of the wall (>10 cm) enclosing the open field. All behaviors were recorded with a series of stopwatches. The open field was cleaned with an unscented disinfectant spray cleaner between each subject to prevent olfactory cues from affecting the behavior of subsequent rats.

b) Morris Water Maze

To examine spatial learning and memory impairments, rats were tested in the Morris Water Maze (Morris, 1981; 1983; Morris et al., 1982). The maze consisted of a 1.6 m diameter circular pool filled (45 cm depth) with water (22^0C) made opaque by the addition of powdered skim milk. A 10 cm^2 platform submerged 2 cm below the surface of the water was placed in the centre of one quadrant of the pool. Latencies to find the platform, total swimming distance and time and distance in each quadrant were automatically recorded using a Videomex-V Image Motion System (Columbus Instruments, Columbus OH). Each animal was given three trials per day for five consecutive days. At the start of

the trial, the animal was placed in the pool at one of four randomly allocated locations. Animals that found the platform within 120 s were allowed to remain on the platform for 10 s; those that did not were manually placed on the platform for the same time period.

On day six, animals were given three visually-cued trials in which the platform was visible (by lowering the water level 2 cm below the top of the platform). These trials were conducted to assess whether the performance of the aged animals could be attributed to visual deficits. Animals received an additional probe trial where the platform was removed from the pool (30 second duration). This trial allows us to compute swim speeds (to assess motor deficits) where the trial duration is constant across all animals, and also permits an examination of the search strategies used by the animals to locate the platform. The pool was divided into four equal quadrants (target, i.e the quadrant previously containing the platform), adjacent clockwise, adjacent counter-clockwise, and opposite quadrants), and the percentage of time spent in each quadrant was computed.

c) Acoustic Startle Response

Startle chambers, equipped with a computer generated program of randomized noise bursts (SRLAB, San Diego Instruments, San Diego, CA) consisted of transparent Plexiglas cylinders (8 cm diameter, 16 cm. length) mounted on a Plexiglas base and placed in sound-attenuating chambers. A speaker mounted in the ceiling of the chamber provided a background noise (70 dB) and the startle stimuli. A piezoelectric accelerator attached to the bottom of the Plexiglas base transduced the startle response. Stabilimeters readings were rectified and digitized on a 0-4095 scale and recorded by SRLAB. The measure of startle amplitude consisted of the mathematical average of 100 1-ms readings collected at stimulus onset.

A test session consisted of placing the rat in the startle chamber for a 5 minute acclimatization period after which they were exposed to a total of 36 (30 msec) stimulus presentations. Stimulus intensity ranged between 80-120 dB, in increments of 5 dB. Each

test subject received 4 presentations at each stimulus intensity. Stimuli were randomly presented with the restriction that there were no more than 2 successive presentations of the same stimulus intensity. Stimuli were presented on a variable time schedule with a range of 5-30 seconds (average=15 seconds).

Stress Sampling

At the end of the experiment, all animals were exposed to a 20-minute period of restraint. Restraint stress was performed between 1000h and 1300h with the use of tubular, plastic restrainers. This period is chosen to avoid the elevated basal B levels and peak HPA responses to stress associated with the dark phase of the cycle (Bradbury et al., 1994). A blood sample (200 µl) was taken immediately before the rat was placed in the restrainer (termed the "pre" sample) and within 1 minute of removal from the home cage. The animals were restrained for 20 minutes, and blood samples were taken at both 5 and 10 minutes after the onset of restraint. Additional blood samples were obtained at the termination of restraint (termed "0") and at 20, 60 and 120 minutes thereafter. Blood samples for B measurement were collected into tubes coated with EDTA placed on ice, and then centrifuged and stored at -20^{0}C until assayed.

Glucose Tolerance Test

Animals were food-deprived for 12 hours overnight and blood was collected via the tail vein at 08:00 h the following day for measurement of basal plasma glucose levels. Two hours later, animals were injected (i.p.) with 2.5 mg/kg of 50% glucose solution (Kalant et al., 1988). Samples were then taken at 15, 30, 45, 60 and 120 minutes post injection. All samples were collected in tubes containing EDTA, centrifuged and stored at -20 ^{0}C until assay. Plasma glucose concentrations were measured by an automated glucose oxidase method using a Beckman Glucose Analyzer 2 (Beckman Instruments, Fullerton, CA).

Radioimmunoassays.

Plasma B and glucose were measured by the radioimmunoassays described in previous chapters.

Statistical analyses.

The results were analyzed by repeated measures and factorial analyses of variance (ANOVA) and simple regressional analysis. Scheffé post-hoc tests were performed when appropriate. Integrated hormone levels were determined with the trapezoidal rule and the data expressed over time of sampling. Group differences at $P<0.05$ were considered significant.

Results

Body Weight and Food Intake

Animal body weights taken once every two to three weeks between 12 and 24 months of age are shown in Figure 71. Body weights were similar at the onset of the study. In general, socially grouped rats had significantly lower ($p<0.05$) body weight compared to all other groups throughout most of the study. Isolated rats had significantly higher ($p<0.05$) body weights throughout the entire study compared to all other groups. Aged control and enriched rats did not differ from each other. Average daily food intake per animals is shown in figure 72. Isolated animals ate significantly less ($p<0.05$) than all other groups on average, and socially grouped rats consumed the most, although this amount was not significant.

Behavioral Parameters

a) Open field behaviors

Latency to emerge into the inner portion of the open field, total time exploring, and time frozen in the open field are shown in figure 73. Latency to emerge into the inner portion is shown in figure 73A. Socially-grouped rats took longer ($p<0.05$) to emerge into the inner portion of the open field when compared to aged control, isolated and young rats. Socially grouped rats took longer than enriched rats, but this was not significant. Compared with enriched rats, socially grouped rats spent significantly less time ($p<0.05$) exploring the open field (fig. 73B). Enriched rats in general spent more time exploring the open field when compared to all other rats, with the exception of young rats. Figure 73C shows that young rats spent significantly less time frozen when compared to any of the aged groups.

b) Morris Water Maze

Figure 74 shows the performance across 5 days on the acquisition of the Morris Water Maze. On day one, young rats had significantly shorter ($p<0.05$) latencies to find the platform when compared to socially grouped, isolated and enriched rats. On day 2, both youngs and enriched had significantly shorter ($p<0.05$) latencies than all other groups, but did not differ from each other. In addition, aged control rats had significantly ($p<0.05$) longer latencies than isolated rats. On day 3, both youngs and enriched had significantly shorter ($p<0.05$) latencies than all other groups but did not differ from each other. On day four, young rats differed ($p<0.05$) from both aged control and isolated groups and enriched showed shorter ($p<0.05$) latencies when compared to isolated rats. Finally on day 5, young rats still showed significantly lower ($p<0.05$) latencies to find the platform when

compared to aged control, isolated and socially grouped rats, but did not differ from enriched rats. The integrated performance over 5 days on the Morris maze is shown in table 15. Aged control rats had a significantly elevated integrated performance on the Morris water maze when compared to youngs and enriched rats at $p<0.05$. Isolated rats had significantly higher overall latencies when compared to youngs and enriched rats at $p<0.05$. Young rats had significantly ($p<0.05$) faster overall learning of the task when compared to all other groups except for the enriched rats. There were no differences on both the visually-cued and probe trials.

c) Acoustic Startle

The response to a series of acoustic startles is shown in figure 75. The response was similar in all groups up until decibel 115, when isolated rats had a significantly ($p<0.05$) higher startle response when compared to socially grouped, enriched and young rats. Isolated and young control rats' startle response remained higher at decibel 120, but this was not significant.

Basal AM and PM Plasma B before, during and following the termination of housing manipulations

Plasma levels of B in the light (AM) and dark (PM) phases of the cycle are shown in figure 76. Plasma B in the AM (panel A) in enriched rats remained the same throughout the study. In contrast, all other groups showed an increase in plasma B over time. At the onset of the study (rats were 12 months of age), all rats had similar levels of circulating B (time point B1). After 7 months exposure to the different housing conditions (age of 19 months, time point B2), socially grouped rats had significantly higher B ($p<0.05$) when compared to all other groups. The other 3 groups had comparable levels of plasma B. At the end of the study (24 months of age, time point B3), all groups continued to show elevations in plasma B, with the exception of the enriched rats.

In figure 76B, basal plasma B sampled in the PM phase of the cycle is shown. At the onset of the study, all rats have similar levels of plasma B. At 19 months of age, socially grouped rats had significantly higher ($p<0.05$) plasma B when compared to the enriched and isolated rats. Isolated rats also had significantly ($p<0.05$) elevated plasma B when compared to enriched rats at time point 2. Finally at 24 months of age enriched rats had higher circulating plasma B when compared to their B at the onset and middle of the study. Socially-grouped rats showed an increase in plasma B over time and had significantly ($p<0.05$) elevated B at time points 2 and 3 when compared to time point 1. Aged control rats had significant ($p<0.05$) elevations in plasma B at time points 2 and 3 when compared to their B at the onset of the study. At time point 3, isolated rats had significant ($p<0.05$) elevations in plasma B when compared to their B at the onset of the study.

Young rats plasma B at time point B3 were also measured (data not shown). In the AM, plasma B levels for young rats were 2.9 ± 1.4 µg/dl and in the PM were 12.5 ± 4.2 µg/dl. Enriched and young rats had similar plasma B levels at time point B3; mean AM plasma B for the enriched rats was 2.3 µg/dl and the PM plasma B was 13.0 µg/dl.

Plasma B before, during and following restraint stress.

Plasma B responses to a 15 minute period of restraint are shown in figure 77. Young and socially grouped rats had the lowest overall plasma B response before, during and following the termination of the 20 minute period of restraint. Isolated rats showed the highest overall response throughout the entire sampling period. Aged control, enriched and isolated rats had plasma B levels that remained elevated and did not return to baseline. Specifically, at 5 minutes following the onset of restraint both socially-grouped and young rats had significantly ($p<0.05$) lower plasma B compared to aged control rats. All rats' B profiles were similar at 10 minutes into stress and at the termination of the restraint. At 20,

60 and 120 minutes following the termination of the stress, isolated animals had significant elevations in plasma B ($p<0.05$) when compared to both socially grouped and young rats. At 60 minutes, however, plasma B levels in isolated rats were significantly higher when compared to young rats only. Integrated levels of plasma B are shown in table 15. Young rats had significantly ($p<0.05$) lower integrated plasma B when compared to both isolated and aged control rats and isolated rats had significantly ($p<0.05$) higher integrated plasma B when compared to socially grouped rats.

Glucose Tolerance Test

Figure 78 illustrates the response to a glucose tolerance test. All rats had similar basal levels of blood glucose. Fifteen minutes following an injection of glucose, isolated rats had significantly lower ($p<0.05$) glucose levels when compared to socially grouped and young rats. At 30 minutes following injection, all rats displayed similar glucose responses. In blood samples obtained 45 minutes after the injection of glucose, enriched rats had significantly higher ($p<0.05$) blood glucose when compared to socially grouped, isolated and age control rats. At 60 and 120 minutes following the injection, enriched rats' blood glucose remained significantly elevated ($p<0.05$) when compared to all other groups. Integrated levels are shown in table 15. Enriched animals had higher ($p<0.05$) integrated blood glucose than socially grouped, isolated and aged control rats and young rats had a significantly ($p<0.05$) elevated integrated blood glucose levels when compared to isolated rats.

Correlations were calculated to assess the relationship between blood glucose and plasma B release and performance on the Morris water maze. These are shown in tables 16-18. Table 16 shows correlational analysis on the integrated blood glucose and restraint-stress plasma B responses and the integrated performance over 5 days on the Morris water maze. The strongest negative correlation existed for the enriched rats, who also showed enhanced spatial learning, followed by the aged control, socially grouped and isolated rats

who had similar performance on both the maze and in response to glucose challenge. Young rats had a very weak correlation. There was a significant positive correlation between restraint-stress integrated plasma B levels and performance on the Morris Water Maze in isolated animals only. Table 17 shows the relationship between AM/PM plasma B at time points B2 and B3 and the integrated performance on the swim maze. A significant correlation was found between plasma B in the AM at time point B2 and the integrated swim performance in socially grouped and isolated rats. There were other significant correlations in the AM or PM in any other groups or at time point B3. Table 18 shows the relationship between all the aged rats' (as a group) integrated Morris water maze performance, integrated response on the glucose tolerance test, integrated plasma B response on the restraint stress and basal B at different time points. A significant negative correlation existed between the aged animals' integrated blood glucose and their Morris water maze performance. A significant positive correlation was found between the aged rats' swim performance and their circulating AM B at the end of the study (time point B3). No significant correlations on any of the measures were found in the young rats.

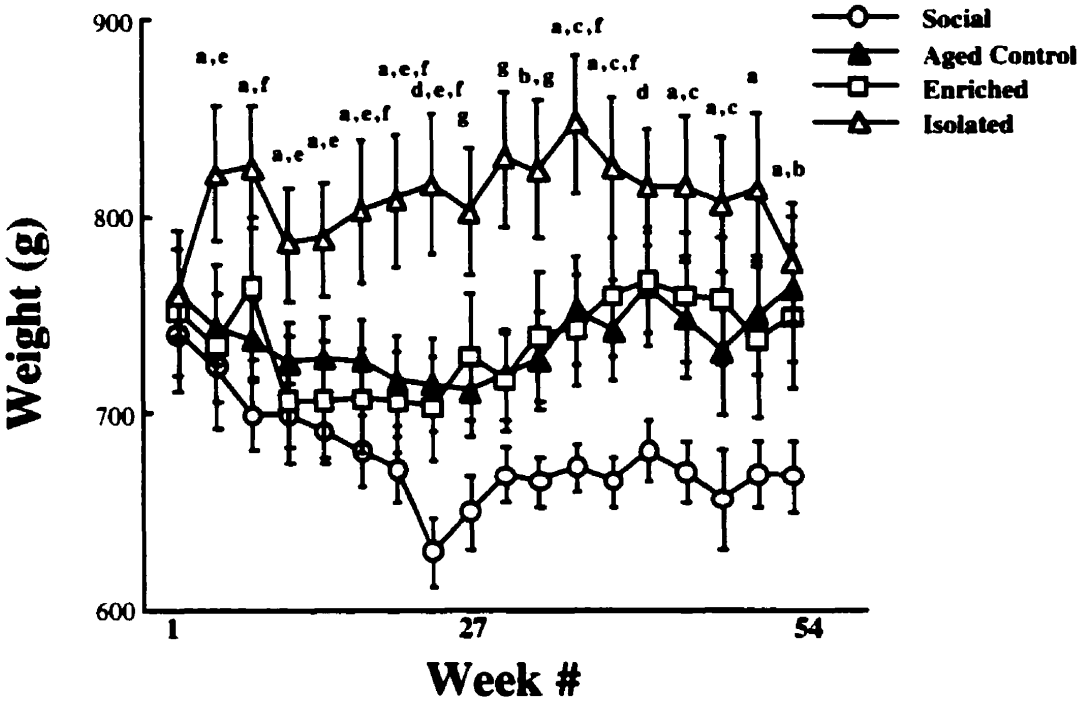

Figure 71. Mean (± SEM) of body weights taken every 2-3 weeks throughout the study. (n=15 per group) a indicates socially grouped vs isolated at $p<0.05$; b indicates socially grouped vs aged controls at $p<0.05$; c indicates socially grouped vs enriched at $p<0.05$; d indicates socially grouped vs all at $p<0.05$; e indicates isolated vs enriched at $p<0.05$; f is isolated vs aged controls at $p<0.05$; g indicates isolated vs all at $p<0.05$.

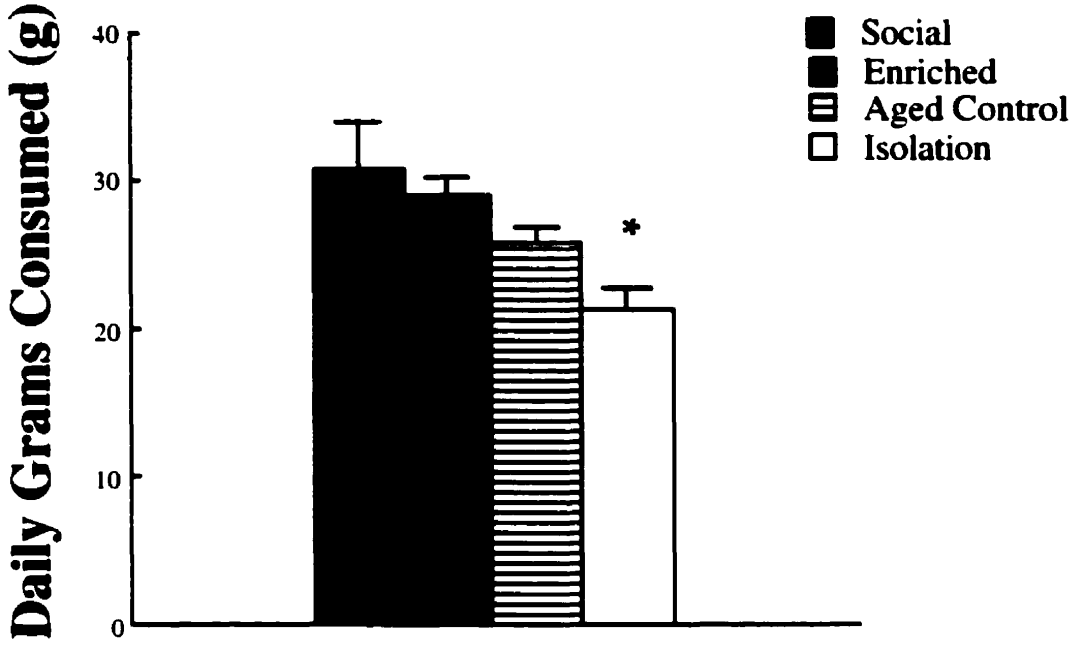

Figure 72. Mean ± SEM average daily food intake. (n=15 per group)* indicates isolated rats differ from all others at $p<0.05$.

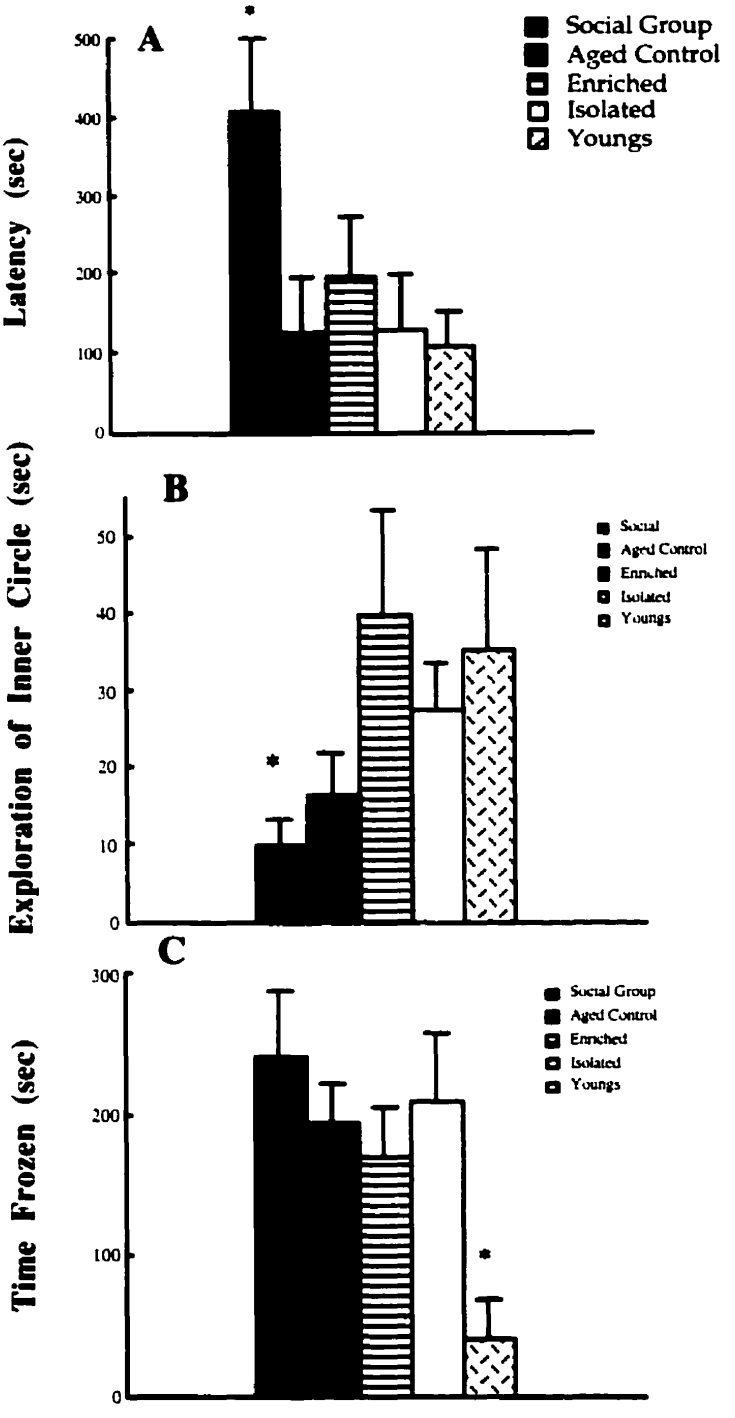

Figure 73. Open field behaviors. Mean ± SEM latency to emerge from outer circle (A), total exploration (B), and time frozen (C) are shown. (n= 10 per group, except youngs=6). In panel A, * denotes socially grouped different from aged control, isolated and young rats at $p<0.05$. In panel B, * denotes enriched different from socially grouped at $p<0.05$. In panel C, * denotes young differ from all others at $p<0.05$

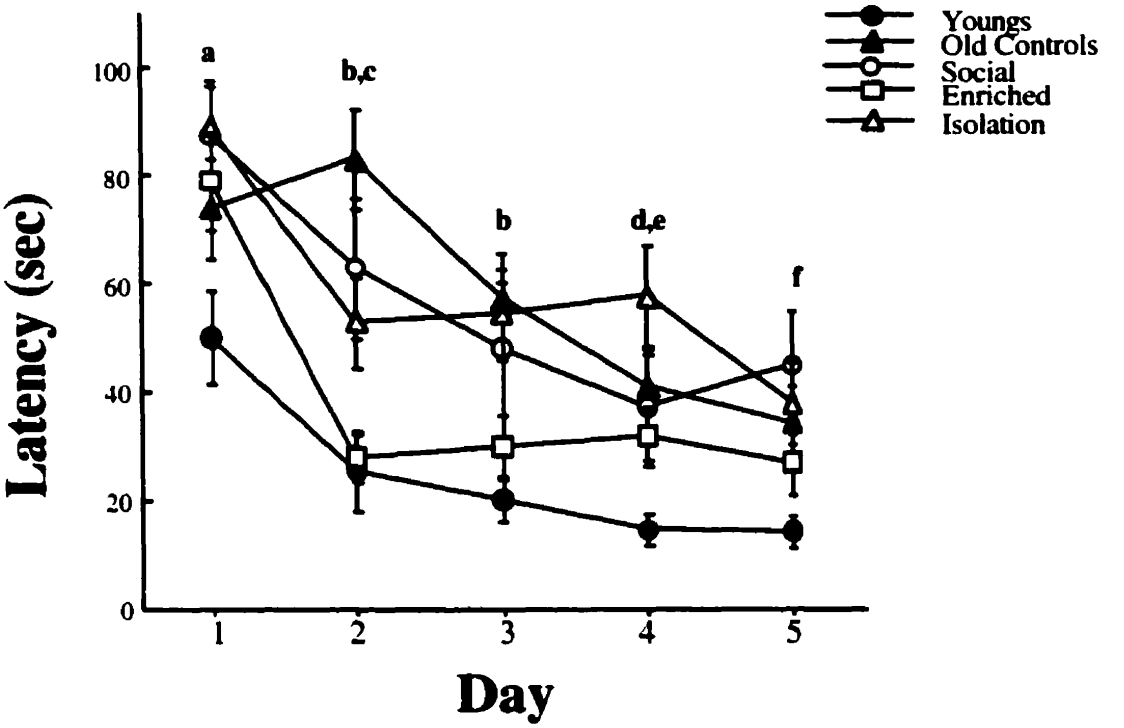

Figure 74. Mean (± SEM) latency to locate the submerged platform on the Morris water maze. (n= 8 per group; youngs = 6) a denotes youngs different from socially grouped, isolated and enriched rats at $p<0.05$. b denotes youngs and enriched different from all others at $p<0.05$. c denotes aged control different from isolation at $p<0.05$. d denotes youngs different from aged control and isolated at $p<0.05$. e denotes enriched differ from isolated at $p<0.05$. f denotes youngs differ from socially grouped, isolated and aged control rats at $p<0.05$.

Table 15. Integrated levels of plasma B, glucose and Morris water maze performance

Experimental Group	n	Integrated Stress Plasma B (μg/dl/min)	Integrated Morris water maze performance	Integrated Blood Glucose (mg/dl/min)
Aged Control	5	56.5± 6.5**	46.9±6.4**	120.4 ± 2.6
Socially grouped	5	30.9 ± 4.5	42.7±6.8+	125.6 ± 9.9
Isolated	5	60.5 ± 16.9*#	45.2 ± 4.3#	106.0 ± 3.5#
Enriched	5	48.9 ± 5.4	28.3 ± 2.6***	146.1 ± 8.2***
Youngs	5	26.0 ± 3.6	18.1± 1.8	130.1 ± 7.7

Mean ± SEM integrated levels of plasma B in response to a 20 minute restraint stress and blood glucose in response to an i.p. glucose tolerance test. (n=5 per group) * denotes isolated rats differ from socially grouped at $p<0.05$; # denotes isolate differs from young at $p<0.05$; ** denotes aged control differ from young at $p<0.05$; *** denotes enriched differs from socially grouped, aged control and isolated at $p<0.05$.

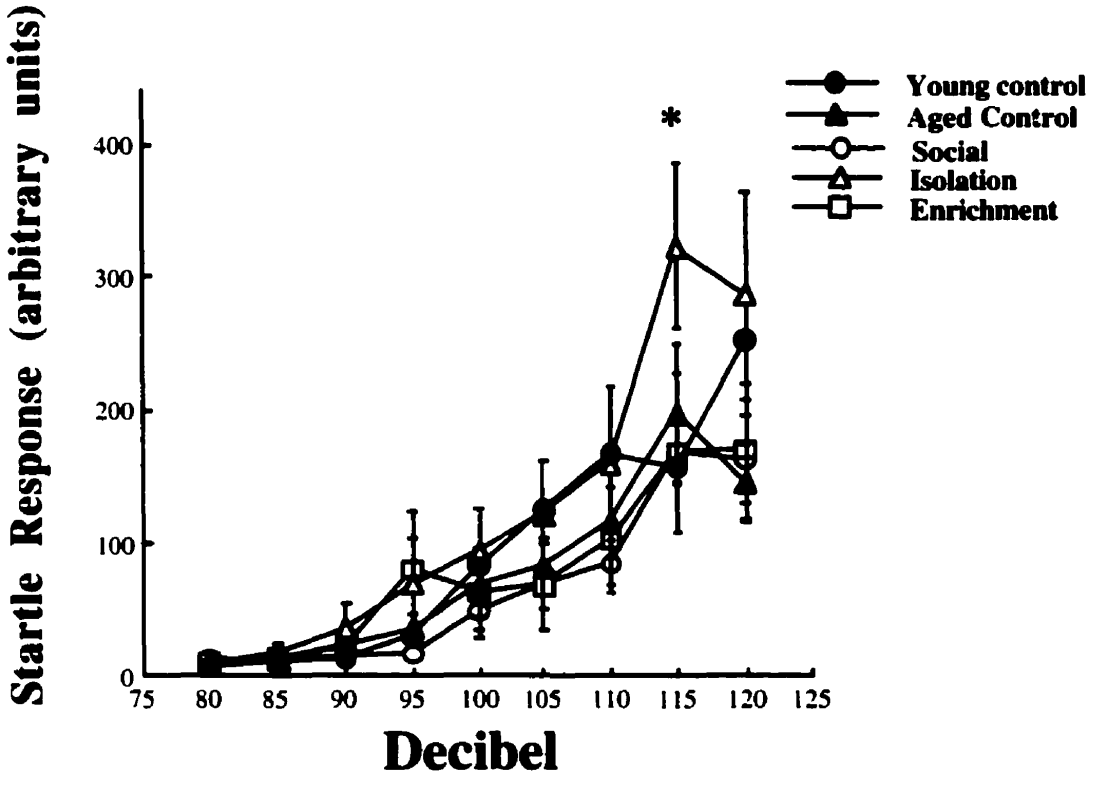

Figure 75. Mean ± SEM response to a series of sound bursts on the acoustic startle. (n= 9 per group; except youngs=6) *denotes isolated different from socially grouped, enriched and young rats.

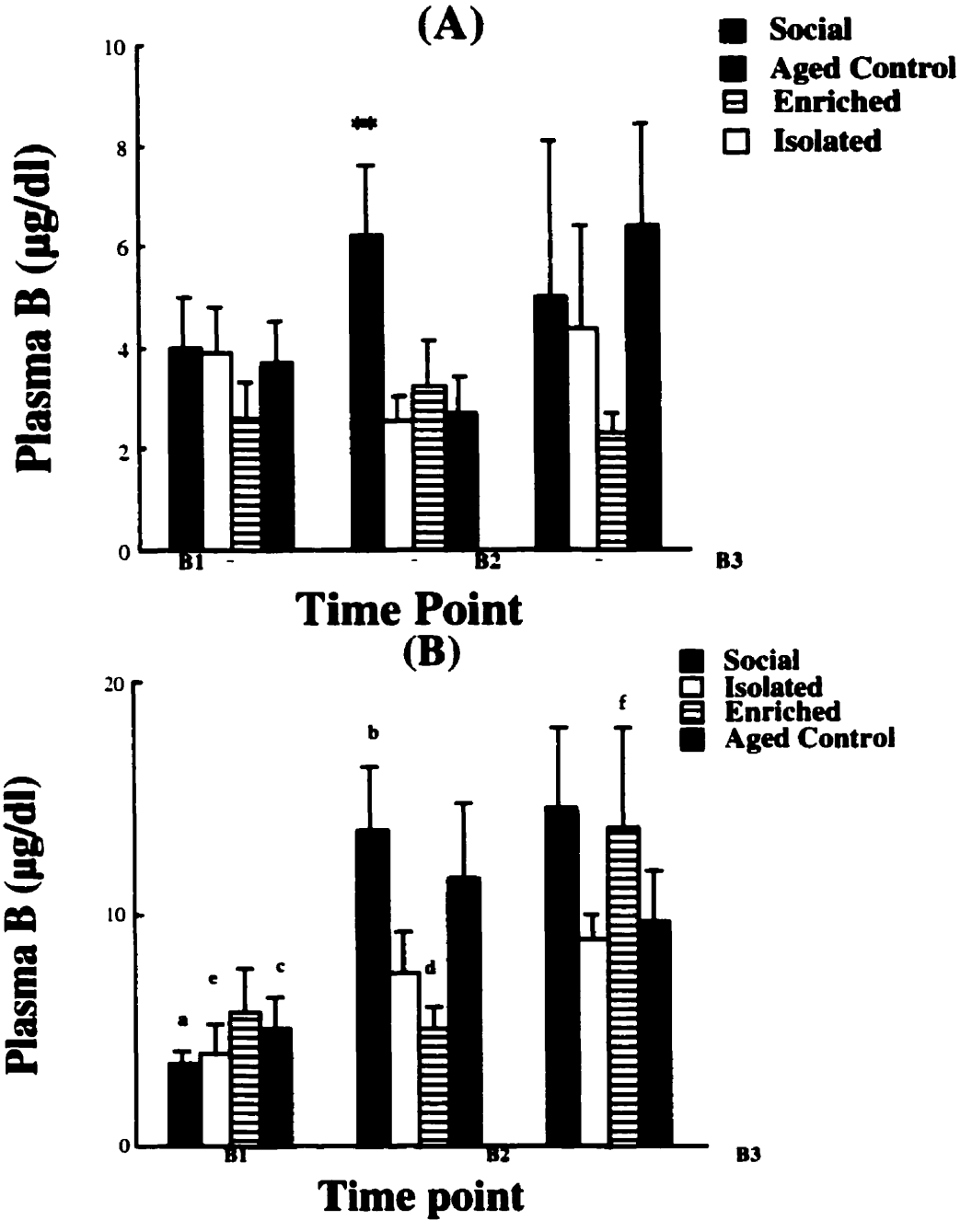

Figure 76. Mean + SEM basal plasma corticosterone sampled in the am (panel A) and pm (panel B) at 12, 19 and 23 months of age. (n= 5-9 per group) In panel A, ** denotes socially grouped rats significantly different from all others at $p<0.05$. In the lower panel B, all differences observed are significant at $p<0.05$. a denotes socially grouped rats at time point one differ from socially grouped rats at time point 2 and 3. b denotes socially grouped rats differ from enriched and isolated rats at time point 2. c denotes aged control rats at time point one differ from aged control rats at time points 2 and 3. d denotes aged control rats differ from enriched rats at time point 2. e denotes isolated rats at time point 1 differ from isolated rats at time point 3. f denotes enriched rats at time point 3 differ from enriched rats at time point 1 and 2.

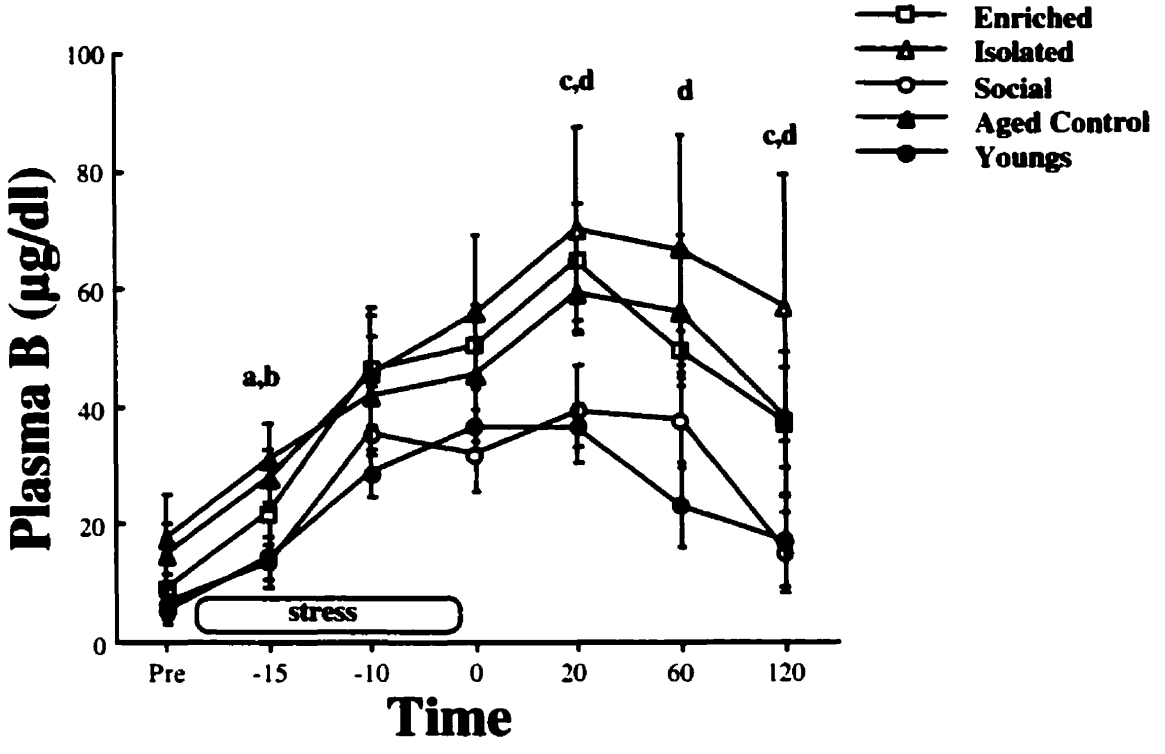

Figure 77. Mean (± SEM) plasma B response before, during and following a 20 minute period of restraint stress. n, = 5 per group a denotes socially grouped differ from aged control at $p<0.05$. b denotes youngs differ from aged control at $p<0.05$. c denotes socially grouped differ from isolated at $p<0.05$. d denotes youngs differ from isolated at $p<0.05$.

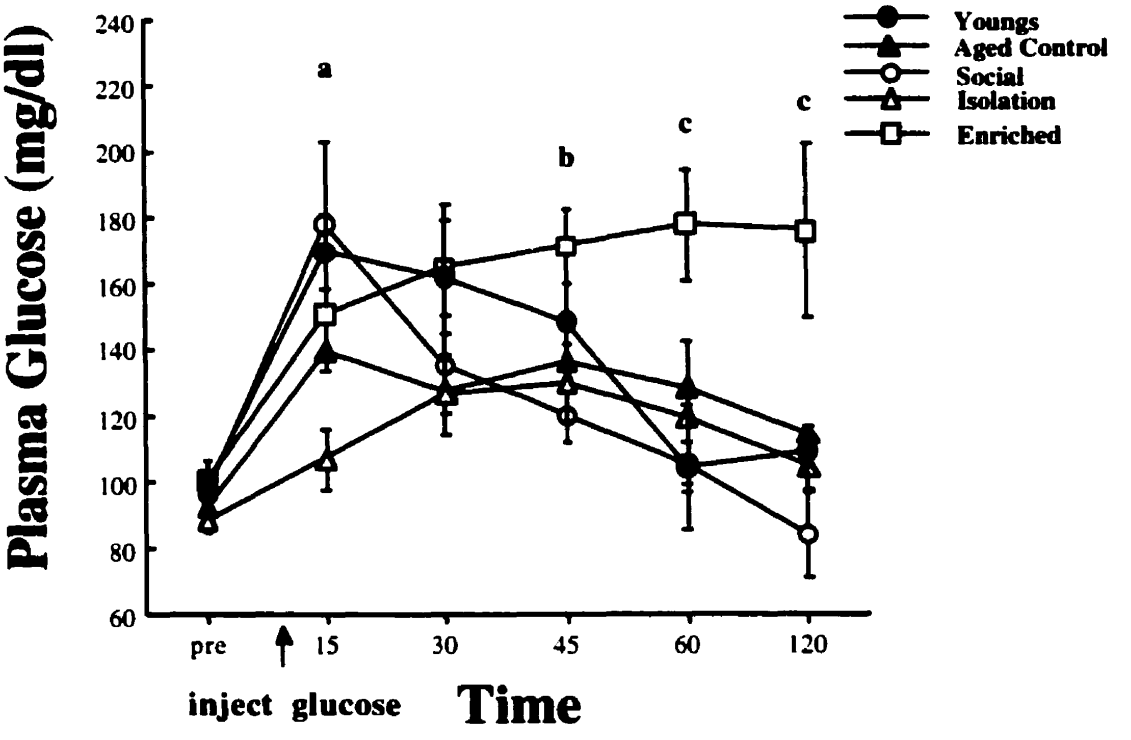

Figure 78. Mean + SEM plasma glucose response to an i.p. glucose tolerance test. n= 5 per group a denotes isolated rats differ from socially grouped and young rats at $p<0.05$. b denotes enriched differ from socially grouped, aged control and isolated rats at $p<0.05$. c denotes enriched differ from all others at $p<0.05$.

Table 16. Relationship between integrated blood glucose levels and integrated performance on Morris water maze in rats

Group	n	R^2 with integrated glucose	P value	R^2 with integrated stress B	P value
Enriched	5	-0.85	0.06	-0.72	0.27
Aged Control	5	-0.49	0.40	-0.48	0.52
Social group	5	-0.43	0.56	-0.72	0.17
Isolated	5	-0.20	0.80	0.92	0.03
Youngs	5	0.15	0.81	0.60	0.31

Table 17. Relationship between basal B (AM and PM) levels and integrated performance on Morris water maze in rats

Group	n	R^2 with AM B2	P	R^2 with PM B2	P	R^2 with AM B3	P	R^2 with PM B3	P
Enriched	9	0.01	0.99	-0.43	0.24	0.15	0.72	0.52	0.23
Aged Control	9	-0.13	0.74	-0.39	0.34	0.001	0.99	-0.46	0.30
Social	5	0.93*	0.02	-0.007	0.99	ns		ns	
Isolated	8	-0.70*	0.05	0.42	0.29	0.51	0.24	0.40	0.44
Youngs	5	ns	ns	ns	ns	0.04	0.94	0.10	0.85

*denotes significance at $p<0.05$

ns denotes insufficient sample size to perform correlations (in the case of youngs, were not yet in the study)

Table 18. Relationship Between Integrated Blood Glucose, Integrated Stress plasma Basal B and Integrated Water Maze Performance in Aged Rats as a group

	Integrated Swim	P value	n
Integrated stress B	0.15	0.54	20
Integrated Glucose	-0.54*	0.02	20
AM B 2	-0.10	0.61	31
PM B 2	0.05	0.80	30
AM B 3	0.41*	0.05	24
PM B 3	0.06	0.80	22

* denotes significant at $p<0.05$

Discussion

These results demonstrate that EE, possibly through its effects on circulating blood glucose and B, significantly attenuated some of the behavioral (open field exploration), cognitive (acquisition of a spatial task) and endocrine (basal and stress plasma B) impairments seen in aging. Specifically, EE increased exploratory behavior in the open-field when compared to socially-grouped rats, enhanced spatial learning and on the Morris Water Maze, dampened AM basal glucocorticoid secretion over time and was associated with a prolonged response to glucose challenge.

Exposing rats to an enriched environment results in a constellation of behavioral changes (Greenough and Volkmar, 1973; Greenough et al., 1986; Mohammed et al., 1986; Rozensweig et al., 1971). Enriched environments increase exploratory activity (Renner and Rozenzweig, 1987; Widman and Rosellini, 1990; Escorihuela et al., 1994; 1995a,b) both in the open-field and on active avoidance tasks. EE produces a decrease in emotionality-related measures, such as defecation, freezing, and/or endocrine responses to novelty/stressors (Escorihuela et al., 1994; Klein et al., 1994), increased exploration/activity in novel situations (Fernandez-Treul et al., 1997; Escorihuela et al., 1994; Renner, 1987; Renner and Rozensweig, 1987; Widman and Rosselini, 1990) and increased learning efficiency or improved ability to cope with aversely-motivated tasks (Cooper and Zubek, 1958; Diamond, 1988; Escorihuela et al., 1994; Greenough et al., 1990; Mohammed et al., 1993; Renner and Rozensweig, 1987). Our data are consistent with these previous findings. Open-field behaviors were similar in isolated and aged control rats, while enriched animals explored more and showed a lower latency to emerge into the inner circle. EE also produced improvements in Morris water maze acquisition; this is consistent with previous work in the field (Mohammed et al, 1990; 1993; Escorihuela et al., 1995; Van Waas and Soffie, 1996; Torasdotter et al., 1996; Van de Weerd et al., 1997). In terms of learning and memory, enriched rats preformed as well as young rats on the Morris water maze across all days, except on day one. By day 2 enriched and young

rats' performance was identical, a trend that remained for the remaining days of testing. Perhaps a complex environment, such as EE, can familiarize the animal with the process of manipulating novel objects and negotiating a home cage that is constantly changing. Having had access to a more complex environment (toys are changed weekly), enriched rats potentially experience less anxiety in a novel situation and freely explore their new surroundings (as evidenced by increased exploration in the open field and superior performance on the Morris water maze).

One potential factor accounting for the behavioral and cognitive changes seen in the enriched animals is an elevation in blood glucose. In response to glucose challenge, enriched animals showed elevations in blood glucose throughout the 2 hours following injection. In a recent report by Messier (1997), animals that received an injection of glucose spent more time exploring in an open field and explored a novel object for a greater amount of time (when compared to a previously-seen object). This suggests that glucose might improve the recall for a previously observed object. In a study by Kopf and Baratti (1996), mice were allowed to explore a novel environment (an open field), on two separate occasions within 24 hours. The difference in the exploratory activity between the first (training) and second (testing) exposures was taken as an index of retention of this habituation task. Postraining i.p. administration of glucose enhanced retention, suggesting that glucose can modulate memory storage of one form of learning elicited by stimuli that are repeatedly presented. When administered just before or after training, glucose enhances memory for recent experiences in healthy elderly people (Craft et al. 1994; Hall et al., 1989; Manning et al., 1990; Manning et al., 1992) as well as in rodents (Wenk, 1989; White, 1991; Gold, 1991). Li et al. (1998) found that a pre-training i.p. glucose injection enhanced memory performance in both a passive avoidance task and on the Morris water maze. A recent report by Manning et al. (1998) suggests that when healthy elderly human subjects are administered glucose immediately before being tested on recall for a passage of text that was read 24 hours earlier, memory storage and retrieval are enhanced. Craft et al.

(1993) demonstrated that individuals with dementias of the Alzheimer's type showed memory facilitation after administration of glucose. Glucose has been found to improve encoding and in some cases, retrieval, in a variety of tasks (Messier and Gagnon, 1996).

The mechanism of glucose's action on cognition includes both peripheral and central effects. Blocking liver efferents has been shown to abolish the effect of glucose on memory (White, 1991) and administration of fructose, which does not cross the blood brain barrier, produces similar memory-enhancing effects as glucose (Messier and White., 1987; Rodriguez et al., 1994). Peripheral actions may require a central neural signal, however, that is produced when glucose is carried into cells via glucose transporter mechanisms (Jay et al., 1990). Glucose easily enters the brain and is its main metabolic fuel (Sieber and Traystman, 1992). Recent microdyalisis experiments showed that when blood glucose concentrations were raised by 300% by a steady infusion of glucose, there was a 200% increase of extracellular glucose in the hippocampus (Harada et al., 1993). A number of groups have also positively correlated circulating plasma glucose and the rate of 2-deoxyglucose disappearance with local cerebral glucose utilization (Vissing et al., 1996). Extremely high or low levels of insulin could affect glucose utilization in those brain regions where insulin receptors are densely represented, namely the hippocampus, the olfactory bulb, and the hypothalamus (Craft et al., 1993). More specifically, glucose has been shown to attenuate the reduction of acetylcholine (ACh) content in the hippocampus produced by atropine injections and glucose increases extracellular ACh following scopolamine injections. The integrity and the state of activity of forebrain cholinergic neurons, in particular the septohippocampal projection have been postulated to play critical functional roles in learning and memory processes (Bartus et al., 1982; Brito et al., 1983; Olton et al., 1986; Durkin, 1989). Glucose also gets taken into cholinergic presynaptic terminals and is transformed into an ACh precursor, acetylcoenzyme A (Reaven et al., 1990; Durkin et al., 1992). A recent report by Ragozzino et al (1998) demonstrated that a unilateral infusion of glucose into the hippocampal formation potentiated the increase in

hippocampal ACh output during spontaneous alternation testing. In marked contrast, when administered to rats at rest, glucose infusions did not modify hippocampal ACh output in the ispilateral or contralateral side, suggesting that glucose may only increase ACh output under conditions in which the activity of the cholinergic neurons is "significantly" altered (Ragozzino et al., 1996). Glucose has been shown to attenuate the amnesia produced by post-training scopolamine in various learning tasks (Messier et al., 1990; Stone et al., 1991).

Other systems may be affected by glucose administration as well. When the dopamine (D2) receptor agonist quinpirole improves performance on a radial maze task, blood glucose levels are elevated. This effect of quinpirole on glucose was due to the release of catecholamines from the adrenal medulla produced by the activation of central D2 receptors. When the D1 agonist SKF 38393 is given there is no concomitant rise in glucose nor is there any improvement on the radial maze task. Glucose can also attenuate the amnestic effects of morphine and this attenuation may be related to the reversal of morphine's action on hippocampal cholinergic function. Talley et al. (1999) have recently shown that peripherally administered glucose attenuated morphine-induced deficits in spontaneous alternation.

In the study by Li et al. (1998) described above, points to another potential mechanism of glucose's action is on cell integrity. Acidic fibroblast growth factor (aFGF), serves to protect cells from degeneration and death, specifically in the hippocampus. In their study, glucose-induced memory facilitation effects were abolished by an icv injection of anti-acidic-FGF antibody. Thus one possible mechanism accounting for the enriched animals enhanced acquisition on the morris water maze could be elevated glucose levels, which may then act on one of the systems mentioned above to improve memory and learning.

When we preformed correlations on the relationship between integrated blood glucose levels integrated Morris water maze performance, we found that across all aged

animals there was a negative correlation, such that the higher their circulating blood glucose, the lower the overall latency to locate the platform on the Morris water maze. Young animals showed a very weak correlation perhaps because elevations in blood glucose may be critical for learning in aged rats but may not be as crucial in young rats. Perhaps the most intriguing finding is that enriched animals had similar resting and peak responses to glucose when compared to the other groups but circulating glucose remained elevated up to two hours following the injection. While it is possible that these animals show impairments in glucose tolerance and clearance, they are not diabetic since their resting fasting glucose is normal (Felig et al., 1995). Performance on the Morris water maze was very weakly correlated with plasma B levels, with the exception of plasma B levels at the last time point in the AM being positively correlated with maze performance. This is not surprising in light of the evidence suggesting that elevations in B are associated with spatial learning deficits, and hippocampal neuron degeneration. What is also interesting is that elevations in B in the middle of the study seem to be a predictor for the performance on the Morris water maze later on.

The differences in endocrine status may explain some of the behavioral findings. When sampled in the AM phase of the circadian cycle, enriched rats showed similar levels of plasma B across time, while the level of basal B in other groups steadily increased as the animals aged. Cognitive deficits following the administration of corticosteroids have been described in experimental animals and humans (reviewed by Lupien and McEwen ,1997). For example, the induction of long-term-potentiation (LTP) is blocked by the administration of chronic or high doses of corticosterone (Dubrovsky et al., 1987; Filipini et al., 1991; Bodnoff et al., 1995). Prolonged exposure to elevated glucocorticoid levels has been shown to promote neuron loss in the hippocampus under a number of conditions (Sapolsky, 1990). Landfield et al. (1978) reported that the degree of pathology in the hippocampus of the aged rat was positively correlated with adrenocortical activity. Adrenalectomy at mid-life, with low-level glucocorticoid replacement, attenuated

hippocampal degeneration and cognitive decline in rats (Landfield et al., 1981). Recent studies in patient populations have suggested a similar relationship in humans. In Cushing's syndrome, which causes prolonged elevations in cortisol hippocampal volume correlates negatively with plasma cortisol levels and positively with percent correct scores on verbal memory tests (Starkman et al., 1992). Lupien et al. (1994; 1998) have found that both acute and chronic cortisol elevations are associated with cognitive deficits in human populations. These studies have also demonstrated that increased cortisol levels are not necessarily associated with aging. In a number of longitudinal studies Lupien's group has found that as individual's age, only a subset of healthy elderlies show cognitive decline. This same subset also uniquely show increased basal cortisol levels (Lupien et al., 1994). Issa et al. (1990) examined HPA function in aged rats that were screened for spatial learning impairments. They found increased basal and stress-induced HPA activity in cognitively impaired aged rats compared to either cognitively-unimpaired aged animals or young controls. We found the same phenomenon in our study: only a subset of animals, namely the EE animals show both decreased basal B levels and no impairment on spatial learning. This suggests that both increased basal glucocorticoid production and the concomitant decline in cognitive function may be required for the emergence of hippocampal aging.

Issa et al. (1990) found significantly lower mineralocorticoid and glucocorticoid receptor density in the hippocampus of aged, cognitively-impaired rats. One possible mechanism that might play a role in dampening B output in our EE animals is elevated levels of GR in the hippocampus (Issa et al., 1990; Jacobson and Sapolsky, 1991; Bradbury et al., 1993). It has recently been shown that GR mRNA expression is induced in the hippocampus following EE (Olsson et al., 1994). This effect is specific to GR, as mineralocorticoid receptor (MR) expression remains unchanged in response to EE (Olsson et al., 1994). Glucocorticoids in excess may be one factor that promotes neuronal dysfunction and loss. It has been shown that upregulation of hippocampal GR may protect

against glucocorticoid-induced neuronal loss (Yau et al., 1999a,b) Therefore an increased density of glucocorticoid receptors in the hippocampus may serve to dampen corticosterone output and as a result spare those neurons in the hippocampus that are sensitive to spatial learning. It is possible that enriched animals have a higher density of GR in those areas involved in mediating negative feedback and that subserve the acquisition of spatial tasks (like the hippocampus).

Another possible mechanism for the enriched animals' lower basal B and sparing of cognitive function is an increase in NGF-IA gene expression in the hippocampus. Olsson's group (1994) have shown that even brief periods of environmental enrichment can cause an increased expression of the immediate early gene, NGF-IA, which mediates, in part, the function of NGF. NGF may then act directly upon GR-expressing hippocampal neurons, since there is evidence to suggest a putative NGF-IA binding sequence in the GR gene promoter (LeClerc et al., 1991). Yau et al. (1996) found that NGF-IA expression was markedly dampened in the hippocampus of aged cognitively impaired rats, who have been shown to have increased B and decreased density of GR (Issa et al., 1990). Environmental manipulations may alter monoaminergic activity as well, and recent evidence suggests that anti-depressants, by enhancing monoaminergic neurotransmission, increase hippocampal GR expression, decrease B and improve cognitive performance (Yau et al., 1995; Rowe et al., unpublished observations). Increased GR expression may then serve to dampen circulating basal B.

Social-grouping produced strikingly opposite effects. Body weight was markedly decreased in socially grouped rats, while food intake increased. Social grouping produced changes in behaviors in the open field: rats were much slower to emerge into the open field and spent less time exploring. Socially grouped rats were slower to acquire the Morris Water Maze, but eventually achieved the same level of performance as the isolated and aged control animals. Basal AM and PM plasma B levels were consistently elevated in socially grouped rats, but were similar to young animals' glucocorticoid response to acute stress.

Finally, although socially grouped rats had an elevated blood glucose response to an injection of glucose, they showed a normal recovery to baseline.

Socially grouped rats also consumed the most food while maintaining the lowest body weight. This is not surprising considering that perhaps one consequence of social grouping is increased physical activity, especially since the housing environment had multiple levels. One of the mechanisms for exercise-induced weight reduction or maintenance in lean individuals is increased energy (caloric) expenditure with compensatory increased intake (Woo et al., 1982; Woo, 1985). In addition, a number of studies in rats suggest that food intake is suppressed in response to brief and acute periods of exercise but long-term, chronic bouts of physical activity are associated with normal or increased food consumption (Davis et al., 1985).With very prolonged exercise, there is progressively less dependence on glucose and increased utilization of fat, resulting in weight reduction or maintenance (Felig et al., 1995).

A number of groups have found that physical exercise can reduce open-field exploration (Bucinskaite et al., 1996; Dishman et al., 1996). Conversely, some recent evidence suggests that chronic forced exercise can have an anxiolitic effect by increasing locomotion in an open field (Dishman, 1997). We found that our socially-grouped rats, who may have been more active, explored the least and had the highest latency to emerge into the inner circle of the open field. This was surprising in light of the recent evidence suggesting that exercise can induce changes in local cerebral glucose utilization (Vissing et al., 1996) and that the incorporation of exercise into one's lifestyle can improve the performance on those cognitive tasks that require effortful processing (Chodzko-Zajko, 1991). In many of these studies, however, the exercise regimen is much more intense and forced (as in treadmill and wheel running) versus our study, where the physical activity was voluntary and spontaneous.

The finding that socially grouped rats show a similar response to glucose challenge when compared to young rats is not surprising. Even mild exercise can increase glucose

uptake from the circulation (Wahren et al., 1971). Low insulin secretion to glucose stimulus has been observed in pancreatic islet cells isolated from exercised rats when compared with sedentary controls (Brandy et al., 1977; Zawalich et al., 1982). Physical activity increases the rate of utilization of all metabolic fuels and increases the sensitivity of glucose utilization by muscle (Langfort et al., 1988).

While a number of groups have used multi-leveled cages with tunnels as enriched environments, it is clear from our results that our enriched rats differed dramatically from our socially grouped rats. In fact, the results obtained from our enriched rats replicate what has been previously found in other enriched rats paradigms. One possibility is that because of the cage set-up (multiple levels, tunnels and 16 rats sharing one large space), a dominance hierarchy might have been established. Blanchard and Blanchard (1990) and Blanchard et al. (1993) describe their visible burrow system (VBS) as model of chronic social stress. This setup consists of a large open field area connected to a series of tunnels and two small compartments (Blanchard and Blanchard, 1990). A dominance hierarchy quickly forms among make rats housed in mixed sex groups in this complex environment. The subordinate males show sustained elevations in plasma B, profound weight loss, impaired testosterone production, thymus involution and adrenal hypertrophy (Blanchard et al., 1993). In addition, although all animals have robust B responses to stress before being housed in the VBS, a subgroup of subordinates show an impairment in their ability to produce the characteristic rise in plasma B when presented with a novel stressor (Blanchard et al., 1993). It is possible that our socially grouped setup was similar to the VBS setup in that most animals were non-responsive subordinates, having elevated plasma B, blunted B responses to acute stress and low body weights. When we examined the body weights of the individual rats in the social group it was clear that there were only two dominant males, while all other males were clustered together as subordinates. This was based on body weight data, which showed that only 2 rats in the social grouping setup gained weight over the 12 months of study, and both of these rats survived the duration of the study. The

average amount of weight lost by the remaining rats was 112 grams, clearly a sign of chronic stress and subordinance and of these subordinate rats, only eight survived the complete duration of study.

Isolated animals had the highest body weight throughout the study with the lowest food intake of all groups. Isolation had no major effect on open-field behaviors, but did impair spatial learning and memory in the Morris Water Maze. In addition, acoustic startle responses were mildly affected by isolation, but only at the highest decibel. Basal plasma B was not significantly altered by isolation, although levels did rise as animals aged. Isolated rats had the highest overall plasma B response to acute restraint, but showed a marked dampened response to glucose challenge.

Not surprisingly, isolated rats had the highest body weight throughout the study. This is most probably due to the fact that isolated rats were the most sedentary since their environment was never changed and they had no opportunity for any interaction with a cage-mate. Since rats will likely sleep most of the day if they are not given any social contact or external stimulation, isolated rats probably had a reduced metabolic demand and did not need to consume as much as other rats. This effect was seen immediately.

Isolated rats show difficulty maintaining the acquisition of the Morris Water Maze task and they are significantly slower than youngs and enriched rats. Social isolation can cause deficits in spatial learning (Einon, 1980; Juraska et al., 1984) but has also been shown to mildly enhance spatial learning (Wongwitdecha and Marsden, 1996) and a recent report by Coudereau et al. (1997) showed that young, isolated mice did not differ from non-isolated on Morris Water Maze and passive avoidance acquisition and retention. This is not entirely surprising since mice are significantly less social than rats and are thus less sensitive to the effects of isolation and the period of isolation was much shorter than the period we imposed. Isolated rats in this study did show subtle increases in their response to startle. A number of groups have found that rearing animals in social isolation can produce increased startle responses and deficits in pre-pulse inhibition (Geyer et al., 1993;

Acri et al., 1995; Varty and Higgins, 1995; Domeney and Feldon, 1998). This is consistent with some previous work suggesting isolation rearing can result in increased psychotic-like behaviors (Kraemer et al., 1984). and increased startle response is consistent with previous work suggesting that rearing rats in isolation display an increase in amphetamine-induced stereotypies as well as an increase in cocaine- and amphetamine-induced locomoter behavior (Boyle et al., 1991; Jones et al., 1988).

Isolated animals showed a blunted response to the glucose injection probably due to absorption of the glucose. Increased adiposity in the isolated rats does not allow for an accurate assessment of tolerance, as absorption of the glucose into the fat cells might be a factor. Another possible mechanism for the blunted response is a low renal threshold for glucose. This is an index of the glucose that is reabsorbed into the kidney tubules and is often proportionate to the amount of glucose filtered and hence to the level of glucose in the plasma. Finally, a high rate of tissue uptake of glucose is possible , although high rates of uptake are usually only seen in lean subjects (Felig et al., 1995) and isolated rats had the highest overall body weight throughout the study.

In sum, manipulating the housing environment can have profound effects on the aging process, but these effects are not uniformly positive or negative. While environmental enrichment may spare some of the cognitive decline and associated hypersecretion of plasma B seen in aging, animals that are environmentally enriched may be at risk of developing glucose intolerance. Social grouping may promote increased B secretion and a certain degree of cognitive decline, but may also dampen the response to subsequent acute stressors and improve glucose tolerance. Isolation is associated with an increase in body weight, an increased latency to acquire the Morris water maze and an increased B response to an acute stressor. Therefore, the ideal environment to promote successful aging would combine physical activity, mental and physical stimulation and social contact. While each of these environments can have beneficial effects on the aging

process, combining components of each environment would promote the most successful aging and perhaps reduce the possible negative consequences of increased age.

GENERAL DISCUSSION

Our findings suggest that both diet and environmental enrichment can affect numerous indices of basal and stress-induced HPA activity and the time when these variables are introduced has a strong impact on the extent of their effects. Manipulating the early environment by introducing short and longer periods of maternal separation has distinct and permanent effects on growth, feeding behavior and insulin dynamics, supporting the notion that there is subtle individual variation in metabolism. These neonatal manipulations also affect individual feeding responses to acute and chronic stress; this variation may be linked to individual differences in HPA activity. In our human studies we have shown that food choices and emotional status are affected by chronic academic stress and these effects may be mediated by dynamic changes in salivary F levels and further influenced by the gender of the subject. In our human aging study we have shown that one potential source for the naturally occurring variation in F in a population of healthy elderlies may be dietary factors; specifically individuals with lower F over years consume less daily calories and fat. This is not entirely surprising as fat is a potent stimulator of HPA activity. One, nine, twelve weeks or 7 months exposure of young rats to a high-fat diet has potent effects on augmenting both basal and acute stress-induced HPA function and on insulin sensitivity. Finally, our last study provided evidence suggesting that environmental enrichment at mid-life has permanent effects on dampening HPA activity and sparing from cognitive deficits, possibly through changes in glucose dynamics.

In chapter one, we showed that the early neonatal environment can exert long lasting changes in growth, food intake and food preferences. While this is a fairly novel finding in rats, there is some anecdotal evidence in humans that suggest an association between the family background and eating disorders. Kinzl's group suggests that negative early life experiences such as a dysfunctional family may contribute to the development of the eating disorder (Kinzl et al., 1994). We know that MS rats receive less licking and grooming and arched back nursing (LG-ABN) (Meaney et al., unpublished observations).

It has also been shown that rats who receive significantly more LG-ABN show reduced behavioral fearfulness in response to novelty compared with the offspring of low LG-ABN (Caldji et al., 1998). In addition, the adult offspring of high LG-ABN show increased central benzodiazepine receptor density in the amygdala and decreased CRH receptor density in the locus ceruleus (Caldji et al.,1998). These areas are known to be involved in the expression of fear and anxiety. In fact, Liu et al. (1997) showed that the offspring of mothers who had exhibited more LG of pups during the first 10 days of life showed reduced plasma ACTH and B responses to acute stress, increased GR mRNA expression and decreased levels of CRF mRNA in the hypothalamus. The emergence of eating disorders usually co-exists with several other disorders, including, but not limited to, anxiety disorder (Herzog et al., 1996), depression (Halmi et al., 1991) and other mood and personality disorders (Wonderlich et al., 1997). Therefore it is not unfounded to propose that the reduction in body weight and food intake seen in MS rats may be part of a constellation of anxiety-related behaviors. Furthermore, the early trauma of maternal separation might have permanent effects on GH and may play a role in the programming of the GH/IGF axis in later life (Kuhn and Schanberg, 1998). Some recent work suggests that chronic suppression of GH secretion in anxious children is associated with growth suppression (Uhde, 1994).

Numerous studies have proposed that indeed, eating disorders are characterized by increased activation of the HPA axis (Laue et al., 1991; Licinio et al., 1996). Anorexia nervosa is characterized by hypercortisolism, increased central CRH and normal or elevated levels of ACTH (Licinio et al., 1996). Again, MS rats show the same profile, specifically increased CRF mRNA and content in the hypothalamus and higher ACTH and B response to acute stress (Plotsky and Meaney, 1993). While the cause of the increased HPA output in MS rats may be maternal factors or some degree of protein-calorie malnutrition (which in and of itself can cause hypercortisolism) or a combination of both, one by-product or consequence of increased B output over the lifespan in the MS rats is alterations in insulin

sensitivity. Therefore these data are consistent with the notion that the effects of the early environment reflect a scenario whereby maternal factors may directly or indirectly program the development of individual differences in growth and carbohydrate metabolism. More work on the glucoregulatory system and the somatotropic axis (IGF/GH) would be necessary and interesting to tease out some of the mechanisms for the MS rats reduced body weight, food intake and emergence of insulin insensitivity.

These data represent a circular and potentially dangerous cascade. Events that occur in early life, such as prolonged maternal separation, may set the stage for the emergence of pathological hormonal milieu: MS is associated with increased B and lower GH, both of which are associated with increased anxiety. Increased anxiety, such as during periods of chronic stress and high levels of B promote body fat deposition and increase fat consumption, both of which serve to further increase B. Ultimately this can result in reduced insulin sensitivity, which further increases fat deposition and levels of lipid in the bloodstream. As the detrimental effects of a high-fat diet (increased FFA, glucose, stress and basal B and insulin insensitivity) are likely mediated through the GCs themselves, superimposing an early trauma, such as MS, may put the animal at a grave disadvantage. The MS rat is metabolically pre-programmed towards these dangerous effects of increased GCs and while MS may not result in increased body fat/weight and fat consumption, ultimately derangements in carbohydrate metabolism can emerge.

We have also shown that insulin sensitivity can be dramatically altered by direct manipulation of the diet. What is critical to ascertain from our high-fat feeding studies is that even a modest augmentation of the fat content in the diet for a relatively short period of time (3 months) can have striking effects on the glucose response to insulin challenge. Again, these changes in insulin sensitivity may be a cause or reflection of changes in HPA activity. High-fat feeding may be considered a form of chronic stress (Tannenbaum et al., 1997), as AM basal and heterotypic stress-induced B (Akana et al., 1992; Scribner et al., 1991) are elevated after just one week of high-fat feeding. High-fat diets cause elevations

in B which may then serve to antagonize insulin's actions, either directly (Reaven, 1988; Brindley et al., 1988; Martin-Sanz et al., 1990) or indirectly, through an elevation in FFA. FFA can reduce the number of both insulin (Anderson and Bridges, 1984) and GC receptors (Vallette et al., 1991), encouraging a state of GC excess and insulin insensitivity. Interestingly, we see the greatest impact of high-fat feeding in young animals maintained on the diet for up to three months. While changes in HPA activity and insulin sensitivity were readily apparent in young high-fat fed rats, there were no changes in food intake and body weight in response to the high-fat diet. When we fed mid-aged rats the diet for 7 months however, we saw similar changes in HPA activity, in addition to changes in body composition and food intake. We also found changes in insulin dynamics except our aged rats showed a striking blunted response to glucose challenge. While fat feeding consistently produces elevations in basal and stress-induced plasma B, prolonged fat feeding for 12 weeks or longer does not produce changes in stress-induced ACTH. Therefore there may be a degree of adaptation in response to more prolonged periods of fat feeding. In fact our receptor binding data would suggest just that. One week of a high-fat diet was associated with decreased GR binding in the hypothalamus while 9 and 12 weeks of fat feeding did not cause any change in binding. While our lack of a difference could be partly explained by the low resolution of our dissection technique, it is entirely possible that levels of B do not increase in proportion to the amount of time on the diet. The possibility of increased adrenal sensitivity to ACTH exists. The response to the chronic stress of a high-fat diet may be part of an adaptation process that does not require all indices of HPA function to be reduced. However the demanding metabolic nature of the stress may require that B release be augmented in response to compensatory rises in insulin.

Twelve weeks of high-fat feeding to young rats produces insulin insensitivity (Tannenbaum et al., 1997). This has been found in a large number of studies (Storlien et al., 1998). A more prolonged regimen of the same high-fat diet, such as in our aging study, does not produce the same effects on insulin, despite significantly increasing

adiposity. When we fed animals high-fat for 12 weeks we didn't find any differences in food intake and body weight when we compared them to our control-fed rats. We did find that 7 months of high-fat feeding was associated with increased body fat and body weight and decreased food intake. It is possible that with the increased adiposity in the fat-fed rats the detection of changes in blood glucose in response to glucose challenge was compromised. Therefore an alternative interpretation for the blunted response to glucose challenge seen in these aged fat-fed rats is that the glucose did not reach its targets at all and was absorbed in the fat. One problem with this interpretation is that both aged fat and aged control rats also had lower basal and stimulated glucose levels. This leads one to consider that perhaps both increasing age and prolonged exposure to fat in the diet are both not necessarily associated with decreased insulin sensitivity. In our study on environmental enrichment we demonstrated that aging in general had no effect on the basal and stimulated glucose response to glucose. In fact, the notion that increased age is associated with increased circulating insulin and glucose is unfounded. Numerous reports suggest that dietary and lifestyle factors are the best predictors for the development of glucose intolerance and insulin insensitivity in rats (Barnard et al., 1995) and humans (Rowe and Khan, 1987), as opposed to increased age. Prolonged fat feeding did cause a substantial increase in adiposity which most likely reflected higher levels of circulating B combined with very low insulin levels (insulin decreases the liberation of FFA).

A substantial amount of evidence suggests that the source of fat in the diet is critical for the development of insulin resistance. Storlien et al. (1996) have reviewed the work in this field and suggest that when n-3 fatty acids and polyunsaturated fats are introduced into the traditional high saturated fat diets, insulin resistance could be virtually eliminated. Thus what may be occurring in our studies is that in the short term high-fat feeding causes a decreased insulin sensitivity, because rats are abruptly switched from a low-fat to a high-fat diet and a number of systems must adjust to the increase in circulating B and the compensatory rise in insulin. However, long-term feeding with corn oil, which is mostly

polyunsaturated fat may have no effect on or may even increase insulin sensitivity (Storlien et al., 1991). A recent study in rats (Picinato et al., 1998) suggests that soybean oil, which is polyunsaturated, caused an enhancement of insulin sensitivity to a glucose stimulus when compared to monounsaturated fats and saturated fats which caused a decrease in insulin sensitivity. Saturated fats also reduced the number of GLUT-2 glucose transporters which, when decreased, accelerate the progression of insulin resistance.

The idea that long-term dietary habits may be protective can be applied to our findings in our elderly population. While glucose tolerance and insulin sensitivity testing was not done on our subjects (which are the most appropriate and relevant tools for the diagnosis of insulin and glucose sensitivity) , none of the subjects we monitored had fasting glucoses in the diabetic range and the average blood glucose value was similar to values reported in young subjects. We did, however find variation in salivary F levels which was associated with an increased calorie and fat consumption. Interestingly, individuals with both increasing and decreasing F with years consumed nearly equivalent amounts of saturated and monounsaturated fat. This is another piece of evidence suggesting that while long-term fat consumption of any kind may increase HPA activity, the consumption of fats that are unsaturated do not necessarily have a detrimental effect on insulin and glucose dynamics. Three months of unsaturated fat consumption in young rats is associated with insulin insensitivity; this may be a temporary state which disappears after more prolonged polyunsaturated fat feeding, which is what we found in both our rat and human aging studies.

In our study on environmental enrichment, an increase in stimulated glucose was positively correlated with an enhanced acquisition of a spatial task. This again underscores the importance of both the age of the animal and when the a particular experimental manipulation is introduced. In an aged rat, the increased stimulated secretion of glucose may be beneficial to its cognitive status, whereas the same glucose dynamic may have detrimental effects in a younger animal. The relationship between glucose and cognition is

intriguing. Glucose may act in the brain by altering neural metabolism, neural activity or neurotransmitter synthesis (Korol and Gold, 1998). One of the first studies done with both young and aged human subjects found that glucose improved the performance on logical memory (contextual verbal information from a paragraph) in elderly subjects only (Hall et al., 1989). Manning's group (Manning et al., 1990; 1992; 1997) extended this same type of finding to other tests of verbal memory (verbatim recall of word lists). Furthermore in an experiment using the same list of words to test different forms of memory, glucose failed to influence implicit memory on a priming task in all subjects but facilitated explicit memory for the word list (Manning et al., 1997). These studies are important for two reasons. First, they clearly show that glucose can specifically enhance memory performance in elderly subjects only and second, glucose enhancement of cognition is restricted to certain classes of memory and cognition. Explicit memory is subserved by the hippocampus (Squire, 1992) and one potential mechanism for a glucose enhancement of hippocampal learning is a potentiation of acetylcholine (ACh) output from the hippocampus (Raggozzino et al., 1996). In fact when glucose does potentiate ACh release from the hippocampus it is during the learning phase, suggesting that animals must be engaged in some kind of learning activity. This is important because the potentiating properties of glucose might only be seen when the animal is stimulated or challenged (i.e. during the acquisition of a spatial task, or when the rat undergoes a glucose tolerance test) as opposed to under basal conditions. In all of our studies with aged rats we saw the same relationship. In both high-fat diet studies and the study on environmental enrichment, the aged animals' acquisition of a spatial (hippocampal) task was negatively correlated with the overall stimulated response to glucose challenge. Interestingly, the environmentally enriched rats, which had a similar cognitive performance to the young rats on the Morris water maze, showed extreme elevations in blood glucose in response to glucose challenge. The young rats' glucose response was not nearly as elevated as the environmentally enriched aged

rats, suggesting that glucose's potentiation of hippocampal-dependent learning is specific to aged animals.

While dietary factors, such as high fat, can influence basal and stress HPA function and carbohydrate metabolism, basal and stress-induced HPA function can influence on food choices. In our study on the early environment we found that the neonatal period can have a tremendous impact on the endogenous preference for fat, protein and carbohydrate. A number of neuropeptides may play a role in the development of these preferences and these neuropeptides may interact with glucocorticoids. Under conditions of both acute and chronic stress food consumption is generally decreased. We also know that H rats secrete less B and ACTH in response to both acute and chronic stressors (Meaney et al., 1995); their hormonal status may also be related to their consumption of particular macronutrients during stress. In response to acute stress, rats will increase their consumption of carbohydrates, perhaps in an effort to increase blood glucose in response to the increased demands of the stressor. Carbohydrate consumption is positively correlated with levels of circulating B; interestingly H rats, which have lower stress-induced levels of B, do not increase their consumption of carbohydrates. The critical conclusion is that H rats may have an endogenous clamp on glucose and insulin levels and as such do not need to consume energy in the form of carbohydrates to increase glucose reserves. Ultimately this clamp results in a normal insulin curve in response to glucose challenge. MS rats, which do consume more carbohydrate, have a significantly reduced insulin sensitivity, which may or may not be diet-induced.

In our young undergraduates we examined chronic stress effects on diet selection. Food preferences changed during the period of chronic academic stress (increased fat consumption) but only in those individuals who showed an increase in F during stressful periods. This is critical in our understanding of the notion of individual variation in the response to stress. While there is substantial evidence suggesting a relationship between GCs, fat consumption and increased adiposity, one cannot overlook the possibility that

there may be additional factors that distinguish our high F/high fat subjects. The bi-directional relationship between fat consumption and stress may be influenced by other factors such as social support, the family background, the processing and perception of the stress itself and the individual's metabolic status (insulin/glucose dynamics, for example).

In that study, subjects tended to decrease their food intake following the termination of the stress. This is important because it demonstrates that the termination of the stressor is not a sufficient stimulus for turning off the actual physiological response to that stressor. In fact the consumption of food was positively associated with the reporting of positive adjectives, suggesting that there is a reward component to eating food. Numerous reports of reduced food intake during or following stressor point to the negative impact that stressors have on reward (Wilner 1997). We also examined gender differences and found that females consume significantly fewer calories before the stressful period when compared to males during this period. There is evidence suggesting that females differ in the cognitive and emotional processing of stressful events (Kirschbaum et al., 1992) and one way females may express this is by reducing their food consumption. In fact our females reported significantly less positive adjectives during the stressful period suggesting that they were feeling more stressed than the males. Females increased saliva F levels during stress may be a result of a dampening of protein consumption which itself can cause increased F and B release (Adlard and Stewart, 1972; Jacobson et al., 1997). These data again provide support for the individual variation in both the emotional and physiological response to stressors and points to the reciprocal relationship between food consumption and GCs. Perhaps our young students, when examined 20 years from now, will show hallmarks of CAD that may have been predicted based on their dietary habits and F profiles.

A conceptual model of the findings of this thesis is summarized in figure 79. The development of the HPA axis is highly sensitive during the early neonatal period, such that manipulations such as prenatal stress, environmental enrichment and handling can alter the

CRH and GR/MR systems. Alterations in these systems may then impact on later B, ACTH and behavioral responses to stress. Furthermore, these alterations may then impact on dietary choices and individual variation in dietary preferences may further modulate HPA axis function. While dietary and long-term consequences of early neonatal manipulations (and their reciprocal effects on each other) may lead (hence the lightening bolt before aging, emphasizing that aging is not an inevitability) to increased B and accelerated aging, environmental enrichment, even as late as mid-life, can prevent or reverse these consequences by dampening the exposure to the highly catabolic GCs.

These studies provide support for the critical role that environmental factors may play in HPA axis function, insulin-glucose axis function, the emergence of individual variation in aging and the interplay between any or all of these factors. The present findings are critical for understanding how factors that affect one system may have a very different impact on that same system if applied at a different stage of life. Further these findings extend our understanding of the aging process in two fundamental ways: first our data lend support for the notion that while an abrupt change in the diet may have serious implications for the development of illness, systems are remarkably flexible in adapting to this change over time. The concept of flexibility can be applied to our study on environmental enrichment which suggests that intervention at a relatively late stage of life can dramatically affect the manner in which the brain ages, by potentially accessing other parallel systems, such as the glucose-insulin axis to prevent the emergence of age-associated pathology. On the other hand we cannot assume an absolute immunity to the effects of increasing age or acute and chronic stress but need to further understand the individual differences in the way that we respond to these challenges. These kinds of investigations would perhaps help to unravel some of the mechanisms that serve to protect some while exposing others to the detrimental effects of chronic exposure to GCs.

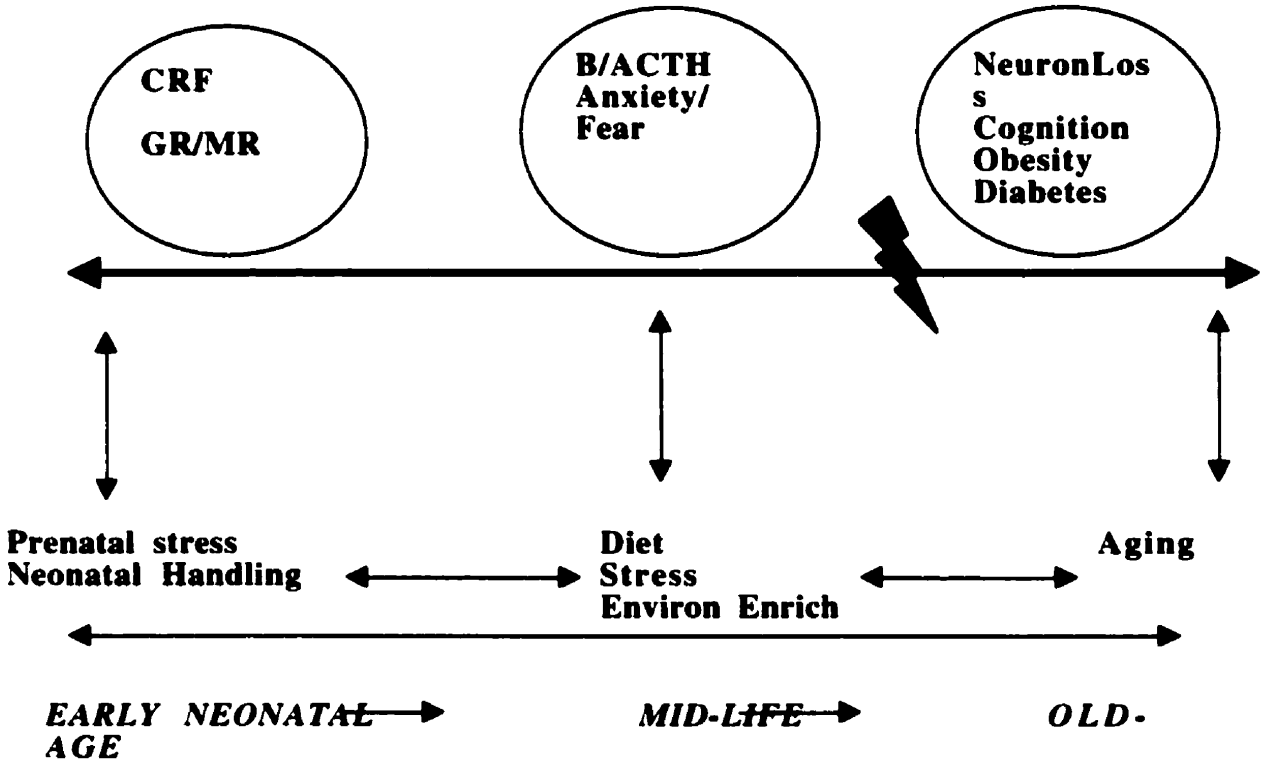

Figure 79. Model of proposed relationship between dietary and environmental factors and the emergence of "aging".

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. Our findings on the effects of neonatal manipulation on later food intake, preferences, body weight gain and insulin dynamics suggest that subtle differences in the neonatal environment can exert profound metabolic changes that may be part of a larger constellation of symptamatology associated with the development of eating disorders and perturbations in the insulin/glucose and somatotropic axes.

2. Chronic academic stress is associated with changes in food intake that are related individual differences in cortisol. These dietary changes may be related to distinct changes in affect in response to stress, lending support for the role that food consumption may play in the emergence of stress-induced changes in F and in affect.

3. Some of the adverse consequences of high fat diets are most likely mediated by elevations in B; not only does this render the animal vulnerable to the development of pathology related to chronic fat, but also exaggerates the response to mild, novel stressors. Hence high-fat diets may themselves be considered as a chronic stress, by elevating basal B production, rendering the animal insulin resistant and facilitating the response to novel stressors.

4. Interestingly, while prolonged high-fat feeding does not seem to impair the acquisition of a spatial task, it does augment the response to novel stressors, increases basal B, and greatly increases adiposity and lowers blood glucose. While the possibility for adaptation to high-fat diets does exist, blunted blood glucose levels in aged animals in general might partly account for any cognitive impairments seen in these aged rats.

5. Elevated fat and calorie consumption are associated with higher plasma and saliva F levels, impaired explicit memory and a reduction in hippocampal volume in elderlies; individual variation in dietary habits over life may be one contributor to the genesis of an individual's F profile. In addition augmented F levels are associated with elevations in triglycerides and cholesterol, suggesting that dynamic changes in F might be one biomarkers for the development of coronary artery disease. Finally, elevated F is also uniquely associated with feelings of stress and depression, again suggesting a relationship between food consumption and mood.

6. Environmental enrichment imposed at mid-life spares animals from cognitive impairment later in life, presumably through a reduction in the overall exposure of the organism to the catabolic GCs and through elevations in blood glucose.

7. Taken together these studies suggest that while both dietary and environmental factors can influence the aging process, systems are remarkably flexible in adapting by accessing parallel systems in times of need. Further, very subtle variations in the rearing environment that lead to stable individual differences can exert a powerful influence on the development of those systems that determine the manner in which one ages.

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Appendix 1: Consent Form, General Questionaire,Schedule, Student Life Stress Inventory, Diet/Sampling/Affective Status Diaries (studies 2 and 5)

Consent Form

I agree to participate in this study on the relationship between dietary intake and salivary cortisol levels. This will involve (a) thoroughly reading and understanding the instructions on how to complete the dietary diary and how to give saliva samples and (b) completing the dietary diary in its entirety, which consists of the Subject Information questionnaire, the Student-Life Stress Inventory, and the actual dietary questions, which will be filled in everyday for two (2) weeks plus five (5) days. I will provide saliva samples five (5) times per day for this same period and will record the times they are taken and other relevant information in the Time of Saliva Sampling and Activity/Mood Dairy section of the dietary diary.

I agree to be as honest and accurate as possible in completing the dietary diary and in providing the saliva samples, as this will ensure more accurate data for the study.

I am aware that the personal information I provide will be kept confidential and will be seen only by the principal investigators at the Douglas Hospital Research Centre.

I am aware that I can withdraw from this study at any time, if for any reason(s), I am unable to continue to participate in it. My signature confirms my understanding of the study and my willingness to participate fully in the study.

_____ _____

Date Signature

Any questions regarding the study can be directed toward Beth Tannenbaum at the Douglas Hospital at 762-3048 from 8:30-4:30 and after at 761-6131 ext. 22361.

SUBJECT INFORMATION (to be done at beginning of study)

(A) *Date of Birth* _____ *Sex* _____

Student status (circle one): **full-time part-time**

(B) *You are currently living (circle one):*

with your parents with a roommate(s) alone

other (please specify)

(C) *Jobs (describe)* _____

Hours per week _____

(D) *Exercise (describe type)* _____

Frequency per week (# days/hrs per day) _____

(E) *Sleep*

hours per day uninterrupted (approx.) _____

usual wake-up time: _____

usual time you go to bed: _____

(F) *Eating Habits*

Usual # of meals per day _____

Appetite (circle one): good fair poor

Recent changes in appetite (please specify and explain):

_____

_____

Foods you avoid _____

why ? (Ex.: Allergies) _____

Your meals are usually prepared by (circle one):

your parent(s) yourself someone else (please specify):

(G) ***Recent weight changes***

loss or gain? _____

# of pounds _____

over what length of time? _____
intentional or non-intentional _____

(H) ***Indicate any vitamins and/or minerals you are taking*: _____**

_____

frequency (number per day) _____

(I) ***Indicate any medication you are taking*: _____**

frequency _____

length of time on medication _____

(J) ***Do you smoke cigarettes?***

If yes , please indicate the number of cigarettes per day _____

Do you use any recreational drugs (i.e marijuana, etc,)? _____

If yes, please indicate how often _____

(K) ***Please indicate any illnesses (short or long-term) or dietary problems (use space below)***

(L) ***Please indicate any special or prescribed diets you are on (use space below)***

(M) ***Academic Schedule for February/March 1997***

exam/assignment/paper	*Due Date*

INSTRUCTION FOR ADMINISTERING THE STUDENT-LIFE STRESS INVENTORY

1. If the students want to participate in the study, give them a copy of the Answer Sheet.

2. Ask them to fill out the information about themselves on the Answer Sheet. e.g., Name, course, age, etc. If the information doesn't apply to them, e.g., number of children just indicate, NA.

Then, ask them to rate their overall stress on the Answer Sheet (Check only one).

3. Then, give each student one set of the 2-page Student-Life Stress Inventory. Read the instructions on the Inventory Sheet.

Ask the students to record each of their answers on the Answer Sheet. There are 51 items (Make sure they do not miss any item).

INSTRUCTIONS ON SCORING THE STUDENT-LIFE STRESS INVENTORY

1. When students' rated their overall stress value on the Answer Sheet before responding to the Inventory, this is their perception prior to their responses and should be treated as such. (You can compare it to their total responses from the Inventory)

2. Add the values of each item and sum them for each category. e.g., there are 7 items in the Frustration category. Add the values for each item and total it for the category. Do the same for the first eight categories. On the ninth category (Cognitive Appraisal), first reverse the values (e.g., 1 is converted to 5, and 5 is converted to 1) Then, sum up the values for Cognitive Appraisal category.

3. You now have scores for each to he nine categories. To get the total score, add up all nine values.

STUDENT-LIFE STRESS INVENTORY

Bernadette M. Gadzella, Ph.D., 1991 Copyright
East Texas State University

This inventory contains statements dealing with student-life stress. Read it carefully and respond to each statement as it has related or is relating to you as a student. Use the 5-point scale which indicates the level of your experiences with:

1= never, 2= seldom, 3= occasionally, 4= often, and 5= most of the time.

Record your responses on the accompanying answer sheet.

I.STRESSORS:

A. As a student:

1. I have experienced frustrations due to delays in reaching my goal.
2. I have experienced daily hassles which affected me in reaching m goals.
3. I have experienced lack of sources money for auto, books, etc.)
4. I have experienced failures in accomplishing the goals that I set.
5. I have not been accepted socially (became a social outcast).
6. I have experienced dating frustrations.
7. I feel I was denied opportunities in spite of my qualifications.

B. I have experienced conflicts which were:

8. Produced by two or more desirable alternatives.
9. Produced by two or more undesirable alternatives.
10. Produced when a goal had both positive and negative alternative:

C. I have experienced pressures:

11. As a result of competition (on grades, work, relationships with spouse and/or friends).
12. Due to deadlines (papers due, payments to be made, etc.)
13. Due to an overload (attempting too many things at one time).
14. Due to interpersonal relationships (family and/or friends expectations, work responsibilities).

D. I have experienced:

15. Rapid unpleasant changes.
16. Too many changes occurring at the same time.
17. Changes which disrupted my life and/or goals.

E. As a person:

18. I like to compete and win.
19. I like to be noticed and be loved by all.
20. I worry a lot about everything and everybody.
21. I have a tendency to procrastinate (put off things that have to be done).
22. I feel I must find a perfect solution to the problems I undertake.
23. I worry and get anxious about taking tests.

II. REACTIONS TO STRESSORS:

F. During stressful situations, I have experienced the following:

24. Sweating (sweaty palms, etc.)
25. Stuttering (not being able to speak clearly).
26. Trembling (being nervous, biting finger-nails, etc.)
27. Rapid movements (moving quickly from place to place)
28. Exhaustion (worn out, burned out)
29. Irritable bowls, peptic ulcers, etc.
30. Asthma, bronchial spasms, hyperventilation.
31. Backaches, muscle tightness (cramps), teeth-grinding
32. Hives, skin itching, allergies.
33. Migraine headaches, hypertension, rapid heartbeat.
34. Arthritis, overall pains.
35. Viruses, colds, flu.
36. Weight loss (can't eat)
37. Weight gain (eat a lot)

G. When under stressful situations, I have experienced:

38. Fear, anxiety, worry
39. Anger
40. Guilt
41. Grief, depression

H. When under stressful situations, I have:

42 Cried
43. Abused others (verbally and/or physically)
44. Abused self (use of drugs, etc.)
45. Smoked excessively
46. Was irritable towards others
47. Attempted suicide
48. Used defense mechanisms
49. Separated myself from others

I. With reference to stressful situations, I have:

50. Thought and analyzed about how stressful the situations were.
51. Thought and analyzed whether the strategies I used were most effective.

ANSWER SHEET TO STUDENT-LIFE STRESS INVENTORY

BERNADETTE M. GADZELLA, PH.D., 1991
EAST TEXAS STATE UNIVERSITY

NAME_____ COURSE_____ AGE _____
MARITAL STATUS: MARRIED _____ SINGLE _____ SINGLE PARENT _____
NUMBER AND AGE OF CHILDREN: NONE _____ CHILDREN _____
COMMUTING: NO _____ YES _____ IF YES, ROUND-TRIP MILEAGE _____
AVERAGE HOURS STUDYING PER WEEK: _____
AVERAGE HOURS WORKING (EMPLOYED) _____

Rate your overall level of stress:
Mild _____ Moderate _____ Severe _____

Respond to each statement in the Student-Life Stress Inventory by recording the level of your experiences on the 5-point scale with 1 = Never, 2 = Seldom, 3 = Occassionally, 4 = Often, and 5 = Most of the time.

	1	2	3	4	5
A. 1.	☐	☐	☐	☐	☐
2.	☐	☐	☐	☐	☐
3.	☐	☐	☐	☐	☐
4.	☐	☐	☐	☐	☐
5.	☐	☐	☐	☐	☐
6.	☐	☐	☐	☐	☐
7.	☐	☐	☐	☐	☐
B. 8.	☐	☐	☐	☐	☐
9.	☐	☐	☐	☐	☐
10.	☐	☐	☐	☐	☐
C. 11.	☐	☐	☐	☐	☐
12.	☐	☐	☐	☐	☐
13.	☐	☐	☐	☐	☐
14.	☐	☐	☐	☐	☐
D. 15.	☐	☐	☐	☐	☐
16.	☐	☐	☐	☐	☐
17.	☐	☐	☐	☐	☐
E. 18.	☐	☐	☐	☐	☐
19.	☐	☐	☐	☐	☐
20.	☐	☐	☐	☐	☐
21.	☐	☐	☐	☐	☐
22.	☐	☐	☐	☐	☐
23.	☐	☐	☐	☐	☐
F. 24.	☐	☐	☐	☐	☐
25.	☐	☐	☐	☐	☐
26.	☐	☐	☐	☐	☐

	1	2	3	4	5
F. 27.	☐	☐	☐	☐	☐
28.	☐	☐	☐	☐	☐
29.	☐	☐	☐	☐	☐
30.	☐	☐	☐	☐	☐
31.	☐	☐	☐	☐	☐
32.	☐	☐	☐	☐	☐
33.	☐	☐	☐	☐	☐
34.	☐	☐	☐	☐	☐
35.	☐	☐	☐	☐	☐
36.	☐	☐	☐	☐	☐
37.	☐	☐	☐	☐	☐
G. 38.	☐	☐	☐	☐	☐
39.	☐	☐	☐	☐	☐
40.	☐	☐	☐	☐	☐
41.	☐	☐	☐	☐	☐
H. 42.	☐	☐	☐	☐	☐
43.	☐	☐	☐	☐	☐
44.	☐	☐	☐	☐	☐
45.	☐	☐	☐	☐	☐
46.	☐	☐	☐	☐	☐
47.	☐	☐	☐	☐	☐
48.	☐	☐	☐	☐	☐
49.	☐	☐	☐	☐	☐
I. 50.	☐	☐	☐	☐	☐
51.	☐	☐	☐	☐	☐

McGill University-Douglas Hospital Research Centre
World Health Organization Collaboration Centre
Department of Psychiatry and Neuroscience

Daily Saliva Samples and Diary

Name: _____

Start Date: _____

Hello and thank you very much for accepting to participate in our study. The following are some important instructions and information on how to give your saliva samples and fill out both the dietary records and information on your emotions. Please feel free to contact us at any time at 762-3048.

GENERAL INSTRUCTIONS

1. For the study, it is preferable that you give us samples and dietary information before, during and after an examination/assignment period. For the additional week or so please give us saliva samples and dietary information during a relatively non-stressful period. Your experimenter will review potential dates with you.

2. Two plastic "Ziploc" bags contain small filter papers (or salivettes) upon which you will give us your saliva samples. One bag contains extra filter papers for taking saliva samples. The other bag contains many filter papers for samples to be given 4-5 times a day for 2 consecutive weeks plus 5 days.

3. Numerous pairs of gloves are provided to you for handling your saliva filters, once they are wet with your saliva. Please use the gloves to put the wet filters into the bags. If you do not want to use the gloves then please be careful NOT to touch the section of the filter paper where you give your saliva sample with your fingers. We have designed these filters with 2 sections: the top part of the section (the smaller portion of the filter) is marked with a small indentation; this is the part of the filter you can touch. The longer part of the filter should not be touched as it is the section where you should give your saliva sample. Please refer to the figure at the end of these instructions for a diagram of the filter.

HOW TO GIVE YOUR SALIVA SAMPLE

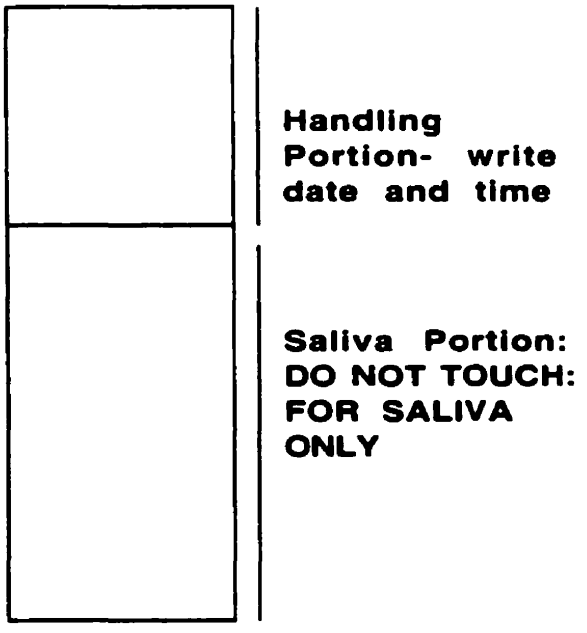
Please take a lot of time to salivate before you place the filter in your mouth. Your mouth should be full of saliva and very wet. The filter paper is divided into two sections: one for handling with your hand and one to actually salivate on. You should never let anything come into contact with the section that you salivate on except for the saliva from your mouth. Anything else (fingertips, dirt, dust, etc...) will contaminate the sample. We have included a little sketch of the filter paper on the next page. After, place the longer section of the filter paper well into your mouth and leave it there until it is entirely wet. If you remove the filter and see that it is not wet enough, please place it back in your mouth and ensure that it gets fully wet. You will know that it is totally wet because the filter turns from white to grey. If you tear the filter, please discard the torn one, take a new filter and give another sample. There are a number of extra filters in the bags for this purpose.

HOW TO DRY YOUR FILTERS

Once the filters are completely wet, you will need to dry them in the air, without having them touch any surface. The best way to do this is to take a small piece of post-it note (or any other sticky paper) and stick the filters on the paper. You must be sure that only the part of the filter that you are supposed to touch (the handling portion) is the part that is stuck to the paper and NOT the part of the filter that has your saliva on it. You may want to cover your furniture with some plastic cover so as to avoid getting the filters on the furniture. You may leave the filter to dry in the air for at least 15 minutes and up to a few hours.

IMPORTANT-Please read the following carefully!!

Please remember to write the date and time of the sample on the part of the filter which is not wet (the handling portion). This is very important so that we can differentiate between individual samples. One hint is to leave a pen on or near the furniture where you are drying your filter papers. This will help to remind you to write the date and time on every sample. If you forget to give a sample, please indicate that on the line, instead of giving us the actual time of the sample. If possible, please staple one day's worth of samples together, once they are dry and ready to be stored in your freezer.



HOW TO STORE YOUR FILTERS

Once the filters are dry, you can pick up the filter (remember to only touch the part that you have not salivated on) and place it (them) in the Ziploc bag(s). Then place the bag in the freezer and once the study is over you should give them to your experimenter. Please take the plastic bag out of the freezer a few hours before you give it to the experimenter so that it may defrost prior. Also, while defrosting, open the bag a little so that the filters do not become humid and too moist. The bag should be dry.

SAMPLING TIMES/DIET DIARIES/ INFORMATION ON EMOTIONS

1)Time of Samples: Please give your saliva samples at 8:00 am, 12:00 pm (noon), 4:00 pm and 8:00 pm. If you rise later than 8:00 am then just mark in the time of your sample and consider that sample as your 8:00 am one. If you cannot do them at this time, then please attempt to give your samples as close as possible to those times. Please indicate the actual time that you have given the sample in the book. At night, if you decide to go to sleep before 8:00 pm (unlikely!!) then please give your sample right before you go to bed and note the time in the book.

2) Illness or Medication/Menstrual cycle: If, during the study, you develop a cold, flu, change your medication or get ill in any other way, please continue to give your samples and kindly note these changes (illness, medication) in your booklets. Please list all medication that you are on at the beginning of the month at the front of the booklet. For female subjects, please indicate the beginning, duration and end of your menstrual cycle. Also, please indicate if you are on the birth control pill. You can list these items in the daily activity section; you may also list these items in the notes section on the daily food record sheets (in the left hand column)

3)Food items: please write in all foods eaten. We would appreciate quantities of what you've eaten (ex.: 1 cup, 1 teaspoon, 1 large, etc...) and we have provided examples of ways in which you can fill in your diet diaries. Please note that if you eat a piece of pie, for example, please draw in the approximate portion eaten (see example in sample diary provided) so that we can estimate the portion eaten. Please fill in all meals eaten plus snacks each day for the duration of the study. If you eat in a restaurant, please give the name of the restaurant and the details of the food ordered. If you skip a meal let us know. Please include components of food items. If you eat a sandwich please break it down onto its parts. For ex: 1/4 lb turkey breast (or two slices) , 2 pieces of lettuce, 2 slices of tomato, 1 tsp. mayo etc...

4) Activity: Please give us an idea of your principal activity for each day (ex.: school, movie, saw friends, housework, job, etc...) and please include any exercise or physical activity you may have done for that particular day. Please indicate either in the notes section of the diet diary sheet or in the daily activity section if you wrote an exam and what your overall perception of the exam was.

5)Information on Emotions: Please circle the emotion that best describes you at each time of the day (for all days) each time you give a sample.

Once again, thank you very much for your participation. If you have any concerns or questions, feel free to contact us at 762-3048. Good Luck!

Beth Tannenbaum and Sonia Lupien

Time of Saliva Sampling and Activity/Mood Diary (to be done daily)

Date _____

(A) Saliva Samples

Recommended sample time	*Actual sample time*
Wake -Up sample	_____
8:00 am	_____
12:00 pm	_____
4:00 pm	_____
8:00 pm	_____

(B)Please describe what you did today in as much detail as possible (this includes any major incidents and illnesses, exams and assignments, personal events.) If possible, please give us some idea about how you felt about the exam or assignment you completed.

(C) Your Emotions and Moods (circle as many as apply); to be done daily, at each time you give a saliva sample.

At 8:00 AM I feel:

happy	**cool**	**rejuvenated**
relaxed	**stressed-out**	**frustrated**
tired	**angry**	**hopeful**
sad	**optimistic**	**disappointed**
positive	**negative**	**energized**
pessimistic	**worrisome**	**confident**
worn-out	**satisfied**	**calm**
down	**pressured**	**up**

At Noon I feel:

happy	**cool**	**rejuvenated**
relaxed	**stressed-out**	**frustrated**
tired	**angry**	**hopeful**
sad	**optimistic**	**disappointed**
positive	**negative**	**energized**
pessimistic	**worrisome**	**confident**
worn-out	**satisfied**	**calm**
down	**pressured**	**up**

At 4:00 PM I feel:

happy	**cool**	**rejuvenated**
relaxed	**stressed-out**	**frustrated**
tired	**angry**	**hopeful**
sad	**optimistic**	**disappointed**
positive	**negative**	**energized**
pessimistic	**worrisome**	**confident**
worn-out	**satisfied**	**calm**
down	**pressured**	**up**

At 8:00 PM I feel:

happy	**cool**	**rejuvenated**
relaxed	**stressed-out**	**frustrated**
tired	**angry**	**hopeful**
sad	**optimistic**	**disappointed**
positive	**negative**	**energized**
pessimistic	**worrisome**	**confident**
worn-out	**satisfied**	**calm**
down	**pressured**	**up**

SAMPLE OF ONE DAY

Journal of the Food I have Eaten on Feb. 3, 1997

MEAL	AMOUNT OF FOOD EATEN	ACTUAL FOOD EATEN
Breakfast	1 slice	whole wheat bread
Indicate time: 8:00 am	1 teaspoon	strawberry jam
Notes:	0.5 cup	low-fat cottage cheese
- had headache, took 2 aspirin	4	prunes
	1 cup	coffee
	1 teaspoon	sugar
	2 tablespoons	2% milk
Lunch	2 slices	white bread
Indicate time: 12:30 pm	5 slices	Cooked turkey breast
Notes:	2 slices	tomato
-ate at a restaurant ("Murray's")	4 tbsp	cooked gravy
	1/4 cup	peas
	1 cup	rice pudding
	1 can	diet coke
Supper	1 cup	cooked spaghetti
Indicate time: 6:00 pm	1 cup	plain tomato sauce
Notes:	1 small slice	french bread
-Took multi vitamin	1 teaspoon	butter
	4 oz	lettuce, tomatos
	2 tbsp.	reduced fat Italian dressing
	1 small scoop	full fat vanilla ice cream
	0.5 cup	grapes
Snack Time: 3 pm	4 1 cup	butter cookies (President's Choice) decaffeinated mint tea
Snack Time: 9:30 pm	1 cup	2% milk

DIETARY DIARY FOR ONE DAY

Journal of the Food/Drink I have Eaten/Drank on_____

MEAL	AMOUNT OF FOOD EATEN	ACTUAL FOOD EATEN
Breakfast		
Indicate Time:		
Notes:		
Lunch		
Indicate Time:		
Notes:		
Supper:		
Indicate Time:		
Notes:		
Snack **Time:**		
Snack **Time:**		