# Glucocorticoid Receptors in the Adrenal Medulla: Characterization, Regulation and Function

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## Abstract

The present thesis has examined in detail the dynamics of adrenomedullary glucocorticoid (GC) receptors at various concentrations of steroid, the regulation of levels of receptor following various treatments, and the regulation of phenylethanolamine N-methyltransferase (PNMT) activity following acute exposure to GCs and various time delays, providing evidence that GC regulation of adrenomedullary catecholamine biosynthesis is more dynamic than was classically thought. We report that adrenomedullary GC receptors are translocated, both in response to nM concentrations of GCs, and in response to higher concentrations of GCs encountered by the glands during stress. We show that long-term increases in cyclic nucleotide second messengers are able to decrease GC receptor binding in adrenal medullary cells, via a mechanism independent of released cortisol, and provide the first evidence that changes in adrenomedullary GC receptor levels are reflected in an alteration in a GC-mediated function, i.e. induction of PNMT. We also provide novel in vitro evidence for the regulation of adrenomedullary PNMT activity, following a necessary lag period, by acute changes in both cortisol and nicotine. In addition, our in vitro studies are supported by our in vivo findings which show increases in adrenal tyrosine hydroxylase and PNMT activity 18h following a single episode of mild acute stress (20 min restraint) in rats.

## Résumé

La présente thèse est une étude detaillée de la dynamique des récepteurs glucocorticoïdiens (GC) des médullosurrénales à diverses concentrations de steroïde, et de la modulation de ces recepteurs suite à des traitements variés. Le controle de l'activité de la phenylethanolamine N-methyltransferase (PNMT) suite à une exposition aiguë aux GCs et les délais variés des réponses obtenues, nous amènent à l'évidence que le controle de la biosynthèse des catecholamines des médullosurrénales est beaucoup plus dynamique qui l'on pensait trandionellement. Nous constatons également que les récepteurs GC sont relocalisés dans le noyau suite à des concentrations élevées de GC rencontrées lors de stress. De plus, nous montrons qu'une augmentation à long terme des niveaus de messagers cycliques secondaires est capable de diminuer la liaison des récepteurs GC dans les cellules médullosurrénales à l'aide d'un mécanisme indépendant de libération de cortisol. Ceci met en évidence que des changements ont lieu au niveau des récepteurs GC, et ceci est réflété par une altération de l'induction de la PNMT par les GC. L'étude apporte également une nouvelle preuve du controle de l'activité de la PNMT médullosurrénalienne après une exposition aiguë au cortisol ou à la nicotine et une attente obligatoire. Enfin, nos études "in vitro" sont, en plus, renforcées par nos découvertes "in vivo" qui montrent des augmentations à 18h de l'activité de la tyrosine hydroxylase surrénalienne et de la PNMT après une simple exposition à un stress moderé et aigu chez les rats.

## Preface

### Statement of Authorship

The following is reproduced in full from the "Guidelines Concerning Thesis Preparation":

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> -Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (eg. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.
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> -In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers.

In all papers presented in the present thesis, Drs. Patricia Boksa and Michael J. Meaney have provided a supervisorial role. Ms. Josie Diorio has provided technical support, in conjuction with the author, for the isolation of the bovine adrenal medullary cell cultures, as well as the maintenance, treatment and harvest of these cultures.

In Chapters II through IV, Ms. Diorio has collaborated with the author in the performance of the enzyme assays for PNMT. In Chapter V, the author obtained the help of Dr. John B. Mitchell and Ms. Seema Bhatnagar for the treatment, stressing and sacrifice of the rats for all experiments except the denervated group of rats. In addition, Ms. Bhatnagar performed the adrenocorticotropic hormone radioimmunoassays and helped the author perform some of the corticosterone radioimmunoassays and adrenal enzyme assays.

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## List of Abbreviations

| | |
|---|---|
| A | adrenaline (epinephrine) |
| ACh | acetylcholine |
| ACTH | adrenocorticotropic hormone |
| ANP | atrial natriuretic peptide |
| AVP | arginine vasopressin |
| B | corticosterone |
| $B_{max}$ | maximal binding capacity |
| BSA | bovine serum albumin |
| CA | catecholamine |
| cAMP | 3',5'-cyclic adenosine monophosphate |
| CBG | corticosterone binding globulin |
| cGMP | 3',5'-cyclic guanosine monophosphate |
| CGRP | calcitonin gene-related peptide |
| CNS | central nervous system |
| CRF | corticotropin releasing hormone |
| DA | dopamine |
| DEX | dexamethasone |
| DMEM | Dulbecco's Modified Eagle's Medium |
| ENK | enkephalin |
| GC | glucocorticoid |
| HRE | hormone response element |
| HTC | hepatoma cells |
| $K_d$ | dissociation constant |
| MMTV-CAT | mouse mammary tumor virus-chloramphenicol acetyltransferase |
| NA | noradrenaline (norepinephrine) |
| PNMT | phenylethanolamine N-methyltransferase |
| RU 38486 | 17ß-hydroxy-11ß,-4-dimethylaminophenyl-17a-propynylestra-4,9-diene-3-one |
| RU 28362 | 11ß, 17ß-dihydroxy-6-methyl-17a-(propionyl)-androsta-1,4,6-triene-3-one |
| SAM | S-adenosyl methionine |
| TH | tyrosine hydroxylase |
| VIP | vasoactive intestinal peptide |

## List of Contributions to Original Knowledge

The following is a listing of original contributions to the literature contained in the present thesis:

1) We have confirmed the presence of glucocorticoid (GC) receptors in the adrenal medulla, using cultured bovine adrenal medullary cells as a model.

2) Using the specific GC radioligand [$^3$H]-RU-28362 and competition studies with various steroids, we have clarified that the subtype of corticosteroid receptor in the adrenal medulla is of the type II (or GC) subtype.

3) A decline in soluble adrenomedullary GC receptor sites and an increase in nuclear uptake of [$^3$H]-dexamethasone (DEX) were found in response to GC levels as low as $5x10^{-8}$ M, suggesting that receptor translocation is occuring at nM levels of steroid.

4) The loss of soluble sites following GC exposure plateaued between $5x10^{-8}$ and $10^{-6}$ M cortisol with a further loss occuring at $10^{-5}$ M and again at $10^{-4}$ M, indicating that translocation was occurring at both low GC levels as well as at higher GC concentrations such as is seen by the adrenal medulla in vivo.

5) The pattern of GC-induced increase in PNMT activity was similar to that seen with GC receptor occupancy; at cortisol concentrations between $10^{-8}$ and $10^{-5}$ M, PNMT induction was at a plateau, with a further increase in activity at $10^{-4}$ M. These data indicate that PNMT activity can be increased by high concentrations of cortisol, over and above the response observed when lower cortisol concentrations are used.

6) The increase in PNMT activity following 3 day exposure to low ($10^{-7}$ M) and high ($5x10^{-5}$, $10^{-5}$ M) cortisol was blocked by the GC receptor antagonist RU 38486, suggesting a GC receptor-mediated event.

7) A short (2h) pulse of GCs, that mimics the time course of physiological elevation of GCs following acute stress, elevated adrenal medullary PNMT activity measured 3 days later.

8) Four day treatment of cells with 8 bromo-cAMP (8 Br-cAMP; $10^{-3}$ M) an analogue of cAMP, or forskolin ($10^{-5}$ M) an activator of adenylate cyclase, decreased soluble [$^3$H]-DEX binding by 55% and 54%, respectively.

9) 8 bromo-cGMP (8 Br-cGMP) treatment decreased [$^3$H]-DEX binding by 31 and 34% at $10^{-5}$ M and $10^{-4}$ M respectively.

10) 8 Br-cAMP and forskolin, but not 8 Br-cGMP increased the concentration of cortisol (presumably released from contaminating adrenocortical cells) in the medium of treated adrenomedullary cells.

11) The presence of bovine serum albumin in the medium of cells treated with 8 Br-cAMP did not prevent the loss in soluble [$^3$H]-DEX binding, suggesting that the effect of 8 Br-cAMP is not mediated indirectly via the release of cortisol.

12) We report a loss in the ability of 8 Br-cAMP or -cGMP treated cells to fully increase the activity of PNMT in response to cortisol, suggesting that changes in adrenomedullary GC receptor levels translate into changes in functional response.

13) We report a decrease in soluble GC binding sites following 4d exposure of medullary cells to $10^{-6}$ M cortisol and to $10^{-5}$ M of the GC receptor antagonist RU 38486, indicating that soluble GC receptor levels can be reduced by both agonist and antagonist.

14) In vitro we show that cortisol pulses ($10^{-4}$ M and $10^{-5}$ M), as short as 15 min, increase PNMT activity measured 2 days following cortisol exposure, with a required lag period of 18h or more.

15) PNMT activity was increased 2 days following brief (2h) exposure to cortisol in concentrations that reach the medulla in vivo ($10^{-6}$ M to $10^{-4}$ M).

16) The increase in PNMT activity following both continuous and 2h pulses of $10^{-5}$ M cortisol was reduced by the GC receptor antagonist, RU 38486.

17) A 2h pulse of nicotine ($10^{-5}$ M) increased PNMT activity with a lag period of at least 18h, while combination treatment of nicotine and cortisol ($10^{-4}$ M) produced significantly higher increases in PNMT compared to either treatment alone, suggesting that there is coordinate control of PNMT activity by hormonal and neural signals.

18) In vivo, we have studied a detailed time course of regulation of rat adrenomedullary PNMT and tyrosine hydroxylase (TH) activity following a single episode of 20 minute restraint stress. Significant increases in adrenal PNMT and TH activity, as well as adrenaline and noradrenaline content, were observed 18h following the beginning of the stress.

19) Suppression of endogenous corticosterone with DEX delayed the stress-induced increase in activity of PNMT, but not TH, suggesting that GCs play an important role in the timing of the regulation of PNMT activity.

## I.1 Introduction

Two classes of hormones secreted by the adrenal gland, the catecholamines (CAs) and the glucocorticoids (GCs), are known to play major roles in stress and homeostasis. During stress, these hormones are released and are responsible for elevating blood glucose, increasing both respiratory and heart rates, and in providing sufficient blood to the brain and to skeletal muscles, thus participating in the stress-induced "fight or flight" response. Regulation of either GC or CA levels in the adrenal gland thus exerts an important influence on the body's response to stress. In mammals, the adrenal medulla is encapsulated by the adrenal cortex, with the medulla releasing CAs and the cortex releasing GCs. As a result of the unique association of the two tissues, there exists an important interaction between these hormones. This interaction results in a GC-mediated regulation of the CA biosynthetic enzyme, phenylethanolamine N-methyltransferase (PNMT), responsible for the synthesis of adrenaline (A).

During homeostasis, GCs were classically thought to play a role in the maintenance of steady-state PNMT levels. Hypophysectomy [with a resultant decrease in adrenocorticotropic hormone (ACTH) and GC secretion] reduced adrenomedullary PNMT activity (Wurtman and Axelrod, 1965, 1966). The decrease in activity was restored to control levels by treatment with GCs or ACTH. Further increases in PNMT activity above homeostatic levels were thought to occur only following extreme taxation of the system, following long-term increases in circulating GC levels in the intact animal. For example, prolonged ACTH administration (Vernikos-Danellis et al., 1968; Simonyi et al., 1985), chronic stress (Kvetnansky et al., 1970), high salt intake in hypertensive rats (Saavedra et al., 1983), or mother-infant separation (Breese et al., 1973), have been shown to increase PNMT activity. In addition, using an in vitro model of the adrenal medulla, bovine adrenal medullary cells, various groups have shown that continuous (18-48h) exposure of chromaffin cells to GCs produced increases in PNMT activity or CA levels, with shorter times of exposure being ineffective (Hersey and DiStefano, 1979;

Kelner and Pollard, 1985; Nawata et al., 1985). Since the duration of increased GC levels during an acute stressor is generally much briefer (<2h) than this, GCs were thought to play little role in the regulation of PNMT activity following more acute exposure of the adrenal medulla to circulating GCs. In addition, GC concentrations reaching the adrenal medulla are in the range of $10^{-6}$ M to $10^{-4}$ M (Kitay, 1961; Peytremann et al., 1973; Jones et al., 1977). Therefore, it was unclear whether receptors for GCs in the adrenal medulla could respond to nM GC levels, as do GC receptors in other tissues, and/or to the higher GC levels seen by the adrenal medulla.

## I.2 Statement of the problem

Therefore, an unresolved issue in the literature, addressed in the present thesis, is the possibility that a short-term elevation in the levels of GCs may be able to regulate the activity of PNMT. Following a single episode of stress, the secreted adrenomedullary CAs, one of which is A, need to be restored to pre-stress levels. Steady-state levels of PNMT may not be sufficient to restore the lost A, and may thus require elevated activity of PNMT in order to replace it with newly synthesized A. Therefore, the general question of the regulation of adrenomedullary PNMT by short-term dynamic changes in GCs was examined in great detail in the present thesis. However, to study this issue, first the dynamics of the GC receptor, reported to be present in the adrenal medulla (Kelner and Pollard, 1985; Nawata et al., 1985), needed to be characterized in greater detail. This receptor has been well studied in various endocrine and neural tissues. In the adrenal medulla, to understand the mechanics of regulation of CA biosynthesis by GCs, a more thorough investigation of adrenomedullary GC receptor dynamics and regulation is essential. Therefore, using bovine adrenal medullary cells in culture, we have characterized the adrenomedullary GC receptor more fully and have studied its regulation by various second messengers in order to understand the basis for the functional outcome of adrenocortical and medullary interactions. Additionally, we have used this model to

study GC regulation of PNMT activity in greater detail, with special attention to the aspect of time of exposure to the GCs. We have examined the time requirements needed to observe such regulation using an exposure regimen such as is found during acute stressors (<2h), and mimicking adrenocortical levels of released GCs. The present thesis provides novel evidence for the regulation of adrenomedullary function by short-term exposure to GCs, and illustrates this finding in both an in vitro culture model and in vivo.

## I.3 Bovine adrenal medullary cells as an in vitro model

Bovine adrenal medullary cells in monolayer culture is an excellent model system that has provided us with many "answers to [our] research questions with a minimum of effort" (Livett et al., 1983). Although many laboratories have used the perfused bovine adrenal to study a number of medullary functions, this preparation would not have been useful for our studies due to the presence of the surrounding cortex. One of the principal reasons for using cultured medullary cells is the ability to control the timing and extent to which these cells are exposed to GCs. This model has been an ideal and simple way to fully explore the characterization, regulation and function of the adrenal medullary GC receptor for several reasons. Firstly, the material used for the isolation of these cells (intact adult bovine adrenals) is readily available from our local slaughterhouse. These cells can be isolated in high yield, and can be maintained in culture for up to two weeks both in serum-containing and serum- and steroid-free conditions (Trifaró and Lee, 1980; Wilson and Viveros, 1981). Cultured medullary cells have been well characterized with respect to their morphology, pharmacology and secretory characteristics, and have been shown to maintain levels of CA stores over time in culture (Trifaró and Lee, 1980; Unsicker et al., 1980; Kilpatrick et al., 1980; Livett et al., 1983). Finally, these cells provide us a possibility to control both the timing and nature of exposure to different exogenous agents, for both short- and long-term studies. The concentration of GCs and the length of time that the cells are exposed to GCs could be easily controlled. This last

point has enabled us to study the central issue of the present thesis, that is the possibility that short-term exposure of medullary cells to GCs can regulate CA biosynthesis over the long-term. The findings we have obtained using this in vitro model have allowed us to turn our investigation of short-term GC exposure on regulation of CA biosynthesis to the in vivo situation, to examine what are the time constraints in situ, given the multiple inputs to the adrenal medulla not present in an in vitro culture model.

In order to more fully understand the basis for the present work, the following is a detailed literature review of the relevant areas of research. The topics covered will include the physiology of stress, as well as the history and physiology of the adrenal gland, one of the major components involved in the stress response. In addition, greater detail will be provided on the nature, regulation and secretion of the contents of adrenal medullary cells, with special attention to the CA biosynthetic enzymes tyrosine hydroxylase and PNMT. The adrenal cortex will also be described in light of the unique association of the cortex and medulla and the resultant interaction between adrenocortical GCs and adrenomedullary CAs. This interaction will also be described in greater detail, both during development and adulthood. In order to appreciate the interaction between GCs and the adrenal medulla, the pharmacology of the GC receptor involved in this interaction will be described, first on a general level, then specifically with respect to the receptor present in adrenal medullary cells. Finally, the dynamics of this receptor will be described to set the stage for our studies which have examined the functional consequence of altering adrenomedullary GC receptors on chromaffin cell function. This literature review should familiarize the reader with much of what is known about the physiological mechanism by which GCs and CAs can and do interact at the level of the adrenal medulla, and can influence the animal's response to stressful stimuli.

## I.4 Stress

Stress has been defined as almost any challege or threat to homeostasis (Munck et al., 1984; Munck and Guyre, 1986). Stressful stimuli are those that create an imbalance in the internal environment, such as heat, cold, or lack of oxygen as well as high blood pressure, bleeding, pain or a strong emotional reaction. The body's homeostatic mechanisms restore the balance of the internal environment. Selye described the response to stress as occuring in several stages (Selye, 1946). The first is the alarm reaction, which is "the sum of all non-specific systemic phenomena elicited by sudden exposure to stimuli to which the organism is quantitatively or qualitatively not adapted" (Selye, 1946). During this stage, the body increases the activity of the sympathetic nervous system, stops digestion, increases the blood sugar level, heart rate, blood pressure and blood flow to the muscles, encompassing the so-called "fight or flight" response. This reaction is initiated by the hypothalamic stimulation of the sympathetic nervous system (leading to release of CAs from the adrenal medulla) and of the adrenal cortex (leading to release of GCs). The resistance stage, which is a long-term reaction to the stressor (i.e. adaptation), mobilizes the internal resources to overcome or escape the stressful stimulus. In the case of long-term stressors, the body may reach the stage of exhaustion, which results in both physical and psychological breakdown. This stage may be caused by overstimulation of the kidneys by mineralocorticoids, leading to potassium loss from cells, as well as by depletion of GCs leading to a decrease in blood glucose levels. Therefore, stress causes activation of both the adrenomedullary and adrenocortical systems, leading to the release of CAs and GCs.

### I.4.1 Stress and CAs

During a stressful stimulus, the nervous input to the adrenal causes the adrenal medullary release of CAs and chromaffin vesicle contents into the plasma. In early studies by Cannon and de la Paz (1911), adrenomedullary A was shown to be released

into the bloodstream following a stressor. The stressor consisted of exposure of restrained cats to a barking dog, a natural stressor for this species. Using a strip of intestinal smooth muscle, whose contraction was known to be inhibited by adrenal secretions, Cannon and de la Paz (1911) showed an inhibition of contraction by blood from the inferior vena cava (just above the opening of the adrenal vessels) collected from "excited" cats. They concluded that the effects of the "excited" blood on contraction were due to an increased content of adrenal secretion. This was supported by their demonstration that removal of the adrenals prior to excitation abolished the ability of the blood to relax the intestinal muscle strip. In addition, varying amounts of A added to blood of non-excited cats produced relaxation of the strip. These studies therefore illustrated that during times of stress, the adrenal gland releases A. In support of this general theory of A release in times of stress, Goodall (1951) showed that of a variety of African species studied, where adrenal A and NA were measured, the hunted animals had predominantly A in their medulla whereas the hunters had predominantly NA. Since hunted animals presumably undergo a more "stressful" lifestyle, this species difference may be related to the ability of GCs to regulate the synthesis of A. Therefore, these studies suggest a link between stress and the adrenal storage and/or release of A.

### I.4.2 Functions of adrenaline

Adrenaline released from the adrenal medulla acts at sites at considerable distances, thus classifying this CA as endocrine. The endocrine sympathoadrenal system is responsible for maintaining homeostatic control over blood pressure, blood glucose levels and oxygen availability. During a stressor, blood glucose levels need to be elevated to provide for increases in energy substrate for a variety of tissues critical for the "fight or flight" response (i.e. the brain, heart and skeletal muscle). Adrenaline stimulates the breakdown of glycogen in the liver to provide more glucose. In adipose tissue, A causes the metabolism of fats to glycerol and free fatty acids, which can be used directly

by the brain and cardiac muscle as fuel or by the liver to manufacture glucose. CAs inhibit the release of insulin, a hormone which promotes the uptake of glucose into tissues (an effect antagonistic to the effect of A), and promote the release of glucagon. CAs also participate in reducing the rate of muscle proteolysis, in increasing the heart rate and rate of breathing, and in shunting the blood to brain and skeletal muscle, thus assisting in the fight or flight response. In addition, CAs relax bronchial smooth muscles allowing for increased availabiliy of oxygen to the blood in times of stress. Therefore, the endocrine actions of CAs help the organism to deal metabolically with stressful stimuli.

### I.4.3 Functions of GCs

During a stressful stimulus, the adrenal gland receives hormonal input, consisting of a 4-5 fold increase in levels of plasma ACTH, causing the adrenocortical release of GCs into the blood. GCs in plasma are reversibly bound to corticosteroid binding globulin (CBG) and other plasma globulins, with approximately 10% of the GCs unbound. It is this unbound steroid that is available for uptake at target tissues and responsible for GC actions. GCs have similar functions to the CAs in their ability to provide for additional glucose for the fight or flight response, due to their acceleration of protein catabolism and conversion of amino acids into glucose. In addition, GCs stimulate the hepatic breakdown of proteins and promote gluconeogenesis, as well as inhibit the secretion of insulin and stimulate the secretion of glucagon (see Munck, 1971; Munck et al., 1984). Therefore during stress, in concert with A, GCs raise the blood glucose levels to provide sufficient energy to tissues critical in the fight or flight response.

Although early physiological hypotheses suggested that GCs function to enhance normal defense reactions against stressful stimuli, GCs were later found to suppress both the immune response and inflammatory reactions (Munck et al., 1984). These GC actions following stress did not appear to serve a protective role for the organism. This

led Munck and his colleagues (1984) to suggest that GCs protect not against the stress itself but against the stress-induced defense mechanisms. These authors suggest that GCs turn off these defense reactions, preventing an overshoot and thus protecting from a possible threat to homeostasis. For example, the anti-inflammatory properties of GCs prevent stress-induced inflammation from being disruptive rather than protective in nature, and resulting in tissue damage following prolonged stress. In addition, Munck et al. (1984) have suggested that following the initial stress and the activation of the appropriate defense reaction, there is a sufficient time delay before the GCs exert their suppressive influence on these defence reactions. Furthermore, Munck et al. (1984) suggested that the negative feedback system is then instrumental in reestablishing the normal levels of GCs "once primary defense reactions have coped with a stress-induced disturbance and glucocorticoids have suppressed the defense reactions".

Munck's theory of the role of GCs in the recovery of the organism following a stressor, and the proposed existence of a time delay, are notions addressed in the present thesis, at least at the level of the adrenal medulla. We have shown that GCs appear to be important for the replenishment of medullary CA stores, via regulation of CA enzymes, following exposure to short-term GCs and a required time delay.

We have discovered that some of the complexity of the interaction between GCs released during a stressful stimulus and the activity of the A-synthesizing enzyme PNMT lies in the time course of exposure of adrenal medullary cells to GCs. However, an interaction between GCs and CAs exists during the resting state as well, and is involved in the homeostasis of the adrenal medulla. GCs are important both for the developmental maturation of chromaffin cell content of CAs and peptides, and for the adult maintenance of these neurohormones. Therefore, in order to understand the basis for the interaction of the adrenal medulla and cortex during a stressful situation, we must first understand the basic physiology of this gland.

## I.5 The adrenal gland

The adrenals are endocrine organs situated above each kidney, and are responsible for the production of steroids and of catecholamines. Steroids are produced in the outer adrenal cortex, a tissue derived embryologically from the mesoderm, whereas CAs are produced from the inner adrenal medulla embryologically derived from the ectodermal neural crest. The adrenal cortex is a true gland with secretions controlled by a humoral input, whereas the medulla is a neuroendocrine transducer with secretions controlled by neural inputs (Pohorecky and Wurtman, 1971). In humans and other mammals, the cortex and medulla form a complex, with the medulla encapsulated by the cortex. Due to the embryological distinction of the two separate tissues which compose the adrenal gland, the cortex has traditionally been studied by the endocrinologist, while the medulla has been the domain of the neuroscientist or pharmacologist (Pohorecky and Wurtman, 1971). The important question, as asked by Dr. Stephen Carmichael (1989), is what is a neural ganglion doing inside an endocrine gland? An answer to this question lies in the interaction between adrenocortical secretions (GCs) and adrenomedullary secretions (CAs).

### I.5.1 History

The first anatomical descriptions of the adrenal (suprarenal) glands were made in 1563 by Bartolomeo Eustacchio, although his descriptions were not published until 1714 by Lancisi (cited in Thorn, 1968). However, the function of the adrenal gland was not described until 1849 when Addison noticed that some of his patients had debilitating symptoms, which at autopsy he ascribed to "a diseased condition of the 'supra-renal capsules'", for the first time suggesting that the adrenal gland was essential for life (cited in Gaunt, 1975 and Allaben, 1982). This was given experimental support in the mid to late 1800s by several French laboratories which showed that removal of the adrenal glands was fatal (reviewed by Gaunt, 1975). Subsequently, a study by Houssay and

Lewis (1923) showed that in dogs, removal of one entire gland and the adrenal medulla of the opposite gland did not impair the health of these dogs, allowing 2 of them to run away from the laboratory in excellent health (Gaunt, 1975). These studies and others of that era (e.g. Hartman, 1922) concluded that although the adrenal cortex was essential for life the medulla was not (Gaunt, 1975). However, the function of this gland remained a mystery for many years as scientists held onto the notion that the adrenal's primary role was the detoxification of circulating humors (Gaunt, 1975; Allaben, 1982). Subsequent studies, using extracts of adrenal cortical tissue (Hartman et al., 1930; Hartman and Brownell, 1930; Swingle and Pfiffner, 1930, 1931) showed that a substance in these extracts could prolong the life of adrenalectomized animals indefinitely, a substance then used successfully in clinical trials for the treatment of Addison's disease (Roundtree et al., 1930). These studies demonstrated the importance of adrenal corticosteroids for the maintenance of a healthy existence, and our current understanding of the physiology, pharmacology and terminology of the glucocorticoids and mineralocorticoids owes much to the advances in the steroid biochemistry field of the 1930s, 40s and 50s (Allaben, 1982).

The influence of adrenocortical secretions on the adrenal medulla and its CA content was suggested in the 1950s by Shepherd and West (1951) and Coupland (1953). The pressor effect of medullary extracts had been known since the late 1800s (Oliver and Schafer, 1894, as cited in Coupland, 1953), the substances now recognized as the amines NA and A (Coupland, 1953). The relative amounts of NA and A were estimated by using a bioassay for the pressor effects of adrenal extracts. This bioassay is based on the effects of an injection of adrenal extracts on blood pressure and nictitating membrane contraction in a spinal cat (Burn et al., 1950). Adrenaline causes both a rise in blood pressure and a contraction of the nictitating membrane, whereas NA increases blood pressure with little effect on the nictitating membrane. Therefore, a ratio of the height of the contraction of the nictitating membrane to the height of the blood pressure rise is

calculated, with higher ratios indicating greater proportions of A. The ratios in unknown samples are compared to the ratios obtained following the injection of standards of known amount of CAs. Using this method in addition to paper chromatography, Shepherd and West (1951) demonstrated that the degree of methylation of NA was correlated with the relative cortical size of adrenals from adult cats, guinea pigs, dogs and humans. Using the same bioassays, Coupland (1953) demonstrated that only NA is responsible for the pressor activity in the dogfish, a species where CA-synthesizing cells are anatomically distinct from adrenocortical tissue, whereas a mixture of A and NA is responsible for the pressor effects in the rabbit and in man, species where the CA-synthesizing cells are surrounded by cortical tissue. In frogs, where CA-synthesizing tissue is partly intra-adrenal without forming a distinct medulla, 55-70% of the pressor response is due to NA. In human and rabbit fetal extra-adrenal CA-synthesizing tissue (para-aortic bodies also known as the 'organs of Zuckerkandl'), the pressor effects are entirely due to NA (Coupland, 1953). Although the bioassay used represented a crude estimation of the relative influence of NA and A, (the effects of adrenal extracts were compared to the effects of standards containing known amounts of the amines), these two sets of studies suggested that adrenal medullary conversion of NA to A was influenced by the presence of adrenocortical tissue. Therefore, the nature of the catecholamine stored within adrenal medullary cells is reflected by the degree of association between the adrenal cortex and medulla (Pohorecky and Wurtman, 1971).

Historically, the interaction between the adrenal cortex and the secretions of the adrenal medulla had been suggested by a number of indirect and correlational studies. With the advent of in vitro model systems, such as medullary cells cultured in monolayer, a more direct investigation of this interaction could be studied. This is the system used throughout most of the studies in the present thesis, in order to study in detail the GC-CA interaction. In the past, these in vitro systems had been used to characterize in detail the morphology and composition of medullary cells. Much of what is known today about

chromaffin cell content, function and secretion was brought about using in vitro medullary cell cultures. The following sections will deal with these topics in greater detail in order to understand the complexity of the interaction between GCs and the adrenal medullary system.

## I.5.2 The adrenal medulla

### I.5.2.1 Catecholamine biosynthesis

CAs are monoamine compounds, containing an amine group and a catechol nucleus (made up of a benzene ring with two hydroxyl groups). In 1939, Blaschko proposed the sequence of biosynthesis of the CAs (dopamine, DA; NA and A). In the adrenal medulla, CA biosynthesis begins with the conversion of tyrosine, an amino acid obtained from the diet, to dihydroxyphenalanine (DOPA) (see Figure I-1). This conversion is catalyzed by the enzyme tyrosine hydroxylase (TH; E.C. 1.14.16.2; Nagatsu et al., 1964), found in the cytoplasm of medullary cells, and is the rate limiting step in CA biosynthesis (Levitt et al., 1965) . The cofactors required for conversion of tyrosine to DOPA are tetrahydropteridine, $O_2$ and $Fe^{2+}$. DOPA is then converted to dopamine, in the presence of vitamin $B_6$ as a cofactor, by the enzyme DOPA decarboxylase (also known as aromatic amino acid decarboxylase; E.C. 4.1.1.28; Blaschko 1939; Christensen et al., 1970) in the cell cytoplasm. Dopamine is then taken up into storage vesicles where it is converted to NA by the enzyme dopamine ß-hydroxylase (DßH; E.C. 1.14.17.1; Levin et al., 1960; Goldstein et al., 1965), requiring $O_2$ and vitamin C as cofactors. In A-storing cells of the adrenal medulla, NA is further metabolized to A by the cytoplasmic enzyme phenylethanolamine N-methyltransferase (PNMT; E.C. 2.1.1.28; Kirshner and Goodall, 1957; Axelrod, 1962), with the help of the cofactor S-adenosylmethionine.

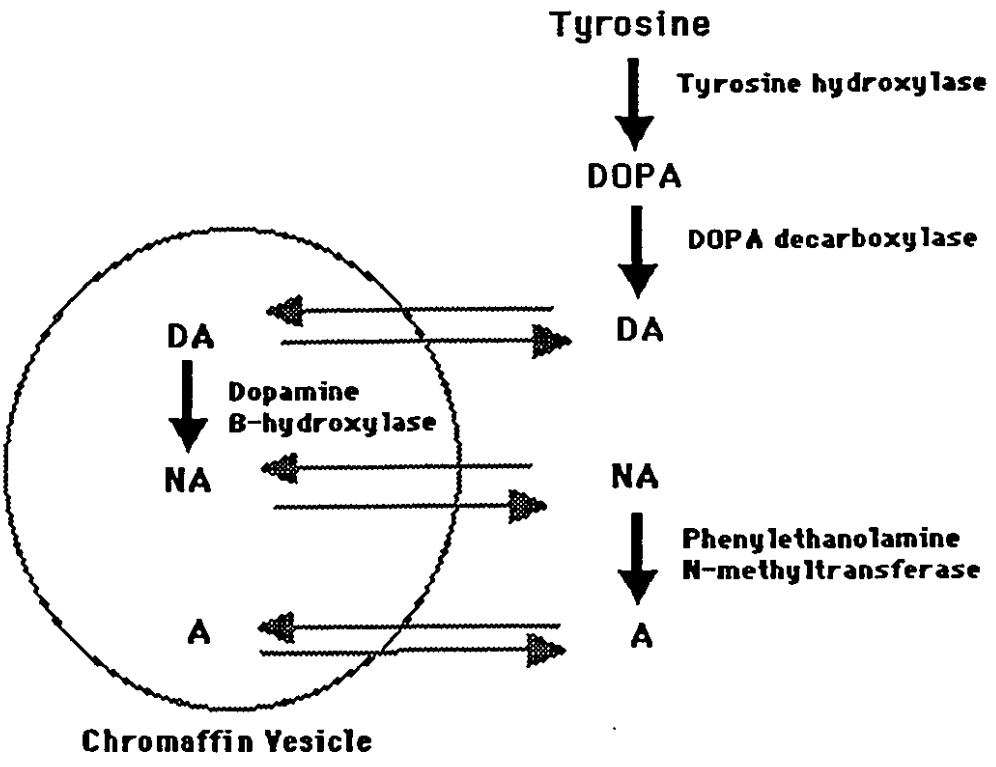


**Figure I-1. Catecholamine biosynthetic pathway.** Taken from Carmichael (1983).

TH, DßH and PNMT are all regulated by both hormonal and neural influences, although each enzyme is predominantly regulated by one or the other influence. In the present study we have focussed on the regulation of PNMT in vitro and in vivo, and the regulation of TH in vivo. We have studied the regulation of TH in vivo since it is this enzyme which is rate-limiting for CA biosynthesis (Levitt et al.; 1965), and have studied the regulation of PNMT due to its role in the conversion of NA to A. Consequently, these two enzymes will be described in greater detail below, with a discussion of the regulation of these enzymes by neural and hormonal influences discussed in later sections.

## Tyrosine Hydroxylase

TH is a mixed function oxidase first identified by Nagatsu et al.(1964). Using both light and electron microscopes, immunocytochemical studies have localized this enzyme to the cell cytosol (Pickel et al., 1975; Nagatsu et al., 1979). TH is the rate limiting enzyme in CA biosynthesis. The evidence for this was provided by Levitt et al. (1965) in studies using an isolated, perfused guinea pig heart preparation. Provided with enough substrate, the formation of NA from dopa or dopamine increased with the amount of precursor available. However, if tyrosine was used as a substrate to measure NA formation, saturation of this substrate was observed, suggesting that it is the conversion of tyrosine, and therefore TH, that is the rate-limiting step in the biosynthesis of CAs. In 1966, Ikeda and coworkers suggested that TH could be inhibited by NA and A, therefore subject to feedback inhibition.

## Phenylethanolamine N-Methyltransferase

PNMT has been localized mainly to the chromaffin cell cytoplasm (Axelrod, 1962; Nagatsu and Kondo, 1974), as well as to a lesser extent within chromaffin vesicles (Brownfield et al., 1985). This enzyme is responsible for the transfer of a methyl group from S-adenosylmethionine to the amine nitrogen of NA (N-methylation; Kirshner and Goodall, 1957). The conversion of NA to A was first demonstrated in 1949 by Buhlbring (Buhlbring, 1949; Buhlbring and Burn, 1949). It was subsequently shown that in addition to NA, PNMT methylates a number of phenylethanolamine derivatives such as normetanephrine, metanephrine and octopamine (Axelrod, 1962). End-product inhibition of PNMT in vitro by both its substrate, NA and its product, A, has been reported by Fuller and Hunt (1965; 1967). Using adrenal gland homogenates from rabbit, rat and bovine species, the $K_i$ (the concentration at which 50% of the activity is inhibited) for inhibition of PNMT was ≈50-100 μM of A. The concentration of A in the adrenal in situ is much higher (≈3-16 mM), a concentration sufficient to cause inhibition

of PNMT in resting adrenals. This led to the proposal that in times of stress, following secretion of medullary A, this inhibition would be released, allowing for an increased rate of formation of A (Fuller and Hunt, 1967). However, PNMT is a cytoplasmic enzyme whereas A is stored within chromaffin vesicles. Therefore, in situ, it is unlikely that PNMT is directly inhibited by A within the cell. Nevertheless, this inhibition of PNMT activity by A in vitro must be acknowledged when considering possible regulation of this enzyme by varying levels of CAs within cytoplasmic extracts used in assays of PNMT activity.

### I.5.2.2 Composition of chromaffin cells

The adrenal medulla makes up 5-10% of the gland by volume (Coupland et al., 1984), and is composed of CA storing chromaffin cells, named for their ability to assume a brown color when stained with a chromic acid solution ("the chromaffin reaction"; from the Greek *chroma* meaning color, and the Latin *affinis* meaning affinity). It was first shown by Eranko (1951, 1952, 1955a, 1955b) using fluorescence microscopy of formalin-fixed sections of adrenal glands from various species, and by Hillarp and Hokfelt (1953) who used potassium iodate histochemistry, that there are two types of cells in the adrenal medulla, A-storing and NA-storing cells. In 1953, Blaschko and Welch, using subcellular fractionation of bovine adrenal medulla homogenates, found that 70% of the A activity (as measured by pressor response in a spinal cat) was present in the particulate fractions. These studies suggested that A was stored in granules which they called "pre-secretory granules" (Blaschko and Welch, 1953). Using osmic-dichromate fixative, Lever (1955) definitively showed that CAs were stored in chromaffin granules, and that these granules were partly lost following a 2h cold stress (suggesting secretion of CAs following stress). An alternate method for fixation of chromaffin cells uses glutaraldeyde, which precipitates NA as a homogenous electron dense complex, thus allowing cells to retain this amine. With this method, A diffuses away from within the

granule leaving only the moderately electron dense granular amine-binding substance. Using this tool, Coupland's laboratory (1964; 1965a) provided evidence for a difference in the histochemical appearance of CA-storing cells, with A-storing cells containing moderately electron dense granules compared to NA-storing cells, which contain intensely electron dense granules (Coupland, 1965a). Due to the difference in the density of A and NA cells, these can be isolated separately on bovine serum albumin (BSA) gradients (Lemaire et al., 1983) and can be used to study selective regulation of each CA.

### Granule contents

Chromaffin granules contain approximately 60% water and a CA content of 2.5 μmol/mg protein (Winkler, 1976). In addition to the presence of CAs stored within the medullary cell granules, these cells contain a variety of minerals, lipids and proteins (for comprehensive reviews see Winkler, 1976; Winkler and Carmichael, 1982). Chromaffin granules contain a large amount of ATP (Hillarp, 1960) and other nucleotides (Rodriguez del Castillo et al., 1988; Castillo et al., 1992), with a molar CA/ATP ratio of about 4.5 (Winkler, 1976). Additionally, chromaffin cell granules contain proteins ("chromogranins", Blaschko et al., 1967), a variety of peptides (summarized in Table I-1; see Pelto-Huikko, 1989 for recent review), trace amines such as serotonin (Brownfield et al., 1985), and the enzyme dopamine ß-hydroxylase (Kirshner, 1959).

The mixture of substances stored within the chromaffin cell granule is secreted by the cell in an exocytotic fashion. It has been calculated that when a chromaffin vesicle fuses with the plasma membrane, it releases about 3 million molecules of CAs, 800,000 molecules of nucleotides such as ATP, 5,000 molecules of chromogranin A, 80 molecules of chromogranin B and several thousand molecules of enkephalin (ENK) precursors and free ENKs (Winkler and Westhead, 1980).

**Table I-1. Chromaffin cell content of various peptides**

| Peptide | Species | References |
|---|---|---|
| VIP | man | Linnoila et al., 1980 |
| | rat | Holtzwarth et al., 1984 |
| | | Hokfelt et al., 1981 |
| | | Kondo et al., 1986 |
| Substance P | man | Linnoila et al., 1980 |
| | rat | Kuramoto et al., 1985 |
| Somatostatin | man | Lundberg et al., 1979 |
| | cat | Vincent et al., 1987 |
| Vasopressin | rat | Hawthorn et al., 1987 |
| and | hamster | Hawthorn et al., 1987 |
| Oxytocin | guinea pig | Hawthorn et al., 1987 |
| | bovine | Hawthorn et al., 1987 |
| ANP | rat | McKenzie et al., 1985 |
| | | Inagaki et al., 1986 |
| ACTH | man | Ito et al., 1981 |
| Dynorphin | pig | Vincent et al., 1984 |
| Enkephalins | rat | Schulzberg et al., 1978 |
| | | Hervoven et al., 1980 |
| | guinea pig | Schulzberg et al., 1978 |
| | cat | Schulzberg et al., 1978 |
| | hamster | Pelto-Huikko et al., 1982 |
| | bovine | Livett et al., 1982 |
| Neurotensin | cat | Lundberg et al., 1982 |
| | | Terenghi et al., 1983 |
| Neuropeptide Y | bovine | Majane et al., 1985 |
| | | de Quidt and Emson, 1986 |
| | horse | Varndell et al., 1984 |
| | cat | Varndell et al., 1984 |
| | rat | Varndell et al., 1984 |
| | guinea pig | Varndell et al., 1984 |
| | mouse | Varndell et al., 1984 |
| CGRP | rat | Rosenfeld et al., 1983 |
| | | Kuramoto et al., 1987 |
| | | Pelto-Huikko and Salminen, 1987 |
| | cat | Pelto-Huikko and Salminen, 1987 |
| | mouse | Pelto-Huikko and Salminen, 1987 |
| Galanin | cat | Pelto-Huikko, 1989 |
| | hamster | Pelto-Huikko, 1989 |
| | mouse | Pelto-Huikko, 1989 |

Summarized from data cited in Pelto-Huikko, 1989. (Abbreviations used: ACTH, adrenocorticotropic hormone; ANP, atrial natriuretic peptide; CGRP, calcitonin gene-related peptide; VIP, vasoactive intestinal peptide.)

## Chromaffin cell peptides

In addition to ENKs present in chromaffin cells, a large number of other peptides have been localized to the adrenal medulla (see Table I-1), in either A or NA cells. ENKs, as well as neurotensin and galanin, are costored in both A- and NA-containing cell types, whereas neuropeptide Y and calcitonin gene-related peptide (CGRP) seem to be stored only in the A cells (see Table 1 of Pelto-Huikko, 1989). Therefore, although there are two subtypes of chromaffin cells (A and NA), within these subtypes there exists a number of different cells which co-store one of the CAs and one or more of the different peptides in Table I-1. A functional role for these medullary peptides has been proposed to include either an intracellular function, a paracrine effect on chromaffin or adjacent cells, a modulation of the stimulation of chromaffin cells by acting on nerve terminals, or a role as a hormone acting at distant sites (Pelto-Huikko, 1989).

To date, the best studied chromaffin cell peptides are the ENKs. These opioid peptides are present in very high levels (several thousand molecules per granule; Winkler and Westhead, 1980), compared to the other peptides in Table I-1. ENKs have been reported to be costored and coreleased with the CAs (Schultzberg et al., 1978; Viveros et al., 1979; Stern et al., 1979; Lewis et al., 1980). The function of released adrenal ENKs have been suggested to include general opioid effects at peripheral sites as well as CNS effects of circulating opioids, since these peptides have been shown to permeate the blood-brain barrier (see Viveros and Wilson, 1983). In addition, adrenal opioid peptides have been suggested to be involved in a naloxone-sensitive stress-induced analgesia since 1) the analgesia produced by prolonged stress is blocked by prior adrenal medullectomy and 2) is potentiated by reserpine administration at doses that increase adrenal medullary opioid peptides (Lewis et al., 1982). Therefore, adrenomedullary ENK synthesis and release during and following a stress response may have an instrumental role in the animal's response to stress.

During stress, the release of the contents of the adrenal chromaffin cells is brought about by an increased firing of the splanchnic nerve innervating the adrenal gland, whereas increased synthesis of CAs and ENKs (see later sections) appears to be influenced by a stress-induced release of GCs. Therefore, incoming stressful stimuli result in both release and subsequent replenishment of chromaffin cell contents. In addition to the GC-induced regulation, the neural regulation of CA biosynthesis has also been addressed in the present thesis. We are reporting that in vitro, activation of nicotinic receptors (which are activated in vivo by neurally released ACh), may alter the GC-induced time course of the regulation of PNMT (chapter IV). In addition, it has been suggested that in vivo, the nerve may be important for maintaining steady-state levels of PNMT (Schalling et al., 1991). Therefore, in order to understand this complex GC-neural interaction, the role of the splanchnic nerve on the synthesis and secretion of CAs will be addressed below.

### I.5.2.3 Innervation of the adrenal medulla

The first evidence for nervous control of adrenal secretion was provided by Dreyer in 1899 (cited in Coupland, 1965c) who showed that following increased stimulation of the splanchnic nerve, adrenal venous blood from a dog caused an increase in blood pressure in a bioassay. The origin of the nerve fibers to the adrenal medulla were suggested by Young (1939) following section of spinal roots in the cat. This work showed a degeneration of nerve fibers reaching medullary cells following sectioning of spinal nerve roots joining the greater and lesser splanchnic nerve (thoracic 7 to lumbar 3 roots; see Figures 7 and 8 of Young, 1939). This suggested that groups of chromaffin cells were innervated separately by different nerve fibers, since the degeneration was limited with any one root transection. It was subsequently shown that these preganglionic sympathetic fibers innervating chromaffin cells were non-myelinated in

nature (Coupland, 1965b). Physiological evidence to support the innervation of the adrenal medulla by the splanchnic nerve was provided in cats (Maycock and Heslop, 1939) where stimulation of the thoracico-lumbar nerves produced increased adrenal medullary secretions as measured in a bioassay (cat nictitating membrane contraction). Therefore, both anatomical and physiological evidence supports an innervation of adrenal medullary cells by the preganglionic splanchnic nerve.

The first demonstration that this nerve was cholinergic came from the classical experiments of Feldberg et al. (1934). In those studies, the effect of acetylcholine (ACh) or splanchnic stimulation was examined in spinal cat adrenal glands, with restricted blood flow in order to measure the pressor effect of medullary secretion on arterial blood pressure. Feldberg and coworkers concluded that the pressor effects of acetylcholine injections (into the coeliac artery) or splanchnic stimulation were due to changes in the output of A, since 1) removal of the glands resulted in a lack of a pressor response to ACh injection and 2) the addition of eserine (to prevent breakdown of ACh) increased the pressor response. These experiments suggested that the release of A from the medulla is due to the splanchnic release of "something indistinguishable from" ACh (Feldberg et al., 1934).

However, these studies could not show that splanchnic nerve stimulation and ACh were directly responsible for A release. Subsequent studies using denervated perfused cat adrenal glands showed that release of CAs from the adrenal medulla occurs in response to application of the nicotine group of cholinomimetics (Douglas and Rubin, 1961b). Nicotine-evoked release of CAs has been shown to be blocked by the addition of the nicotinic antagonist hexamethonium, both in cat adrenals perfused in situ (Douglas and Poisner, 1965; Collier et al., 1984) and in perfused bovine adrenals (Wilson and Kirshner, 1977), thereby confirming the cholinergic nature of CA release. The release of the secretory contents of chromaffin cells in response to an excitatory stimulus has been termed "stimulus-secretion coupling" analagous to the skeletal muscle "stimulus-

contraction coupling" (Douglas and Rubin, 1961b). In addition, using the denervated perfused cat adrenal preparation, it was reported that calcium was necessary for ACh to produce appreciable CA release, whereas removal of all sodium and potassium from the perfusion medium did not prevent the ACh-induced CA release (Douglas and Rubin, 1961a; 1963). Additionally, more recent studies have demonstrated that, following splanchnic stimulation, the release of CA from the cat adrenal is well correlated with the release of ACh, reflecting a linear input-output relationship (Collier et al., 1984).

### I.5.2.4 Splanchnic nerve contents

Although the splanchnic nerve release of ACh seems to be primarily responsible for adrenal medullary CA release, this release may be modulated by the variety of peptides that have been immunocytochemically localized in the nerve fibers innervating the medulla (for review, see Pelto-Huikko, 1989). Although there are species differences, Pelto-Huikko (1989) has shown that there is selective localization of peptidergic neurons to either A- or NA-storing cells, suggesting differential release of either CA depending on the incoming stimulus. For example, in the rat, there seem to be three populations of preganglionic neurons containing either 1) ACh only (innervating both A and NA cells), 2) ENKs only (innervating A cells) or 3) both ACh and ENKs (innervating A cells). In the cat and mouse, in addition to ENKs and ACh, neurotensin has been found in nerve terminals, innervating NA cells. In all species, galanin was found in nerve terminals mainly at A cells, and in the rat, CGRP nerves (that co-localize substance P) innervated PNMT-immunoreactive (A) cells (Pelto-Huikko, 1989). Neuropeptide Y (NPY) fibers and VIP fibers (Holzwarth, 1984) in the medulla appear to contact some chromaffin cells with no specific relation to the two cell types, with these fibers themselves being innervated by enkephalin fibers (for NPY) or CGRP/substance P fibers (for VIP) (Pelto-Huikko, 1989). These studies suggested that the adrenal medulla is not only under cholinergic preganglionic control but that CA and peptide secretion from chromaffin cells

may be under multiple regulation by peptidergic and cholinergic contents of the nerve fibers.

### I.5.2.5 CA secretion

#### Nicotinic and muscarinic control

Cholinergic stimulation of adrenal CA release appears to have both a nicotinic and muscarinic component. Early pharmacological studies by Dale (1914) served to classify the "muscarine-like" and "nicotine-like" actions of choline and its derivatives on arterial blood pressure of the cat. Small doses of atropine (a muscarinic antagonist) abolished the depressor effect of these drugs on blood pressure ("muscarine-like" action), and demonstrated that the stimulant effect of ACh on the adrenal medulla is "nicotine-like". However, Feldberg and coworkers (1934) later demonstrated the presence of a "muscarine-like" effect on cat adrenal medullary secretion since an arterial injection of muscarine stimulated to some extent the output of CAs from the medulla. Therefore, in the cat, the release of adrenal CAs can be evoked by both muscarinic and nicotinic actions.

Although early work (Feldberg et al.,1934; Douglas and Rubin, 1961b; Douglas and Poisner, 1965) suggested both a muscarinic and nicotinic component to the cholinergic stimulation of CA release in the cat, the relative importance of each receptor type appears to be species-dependent. It had been reported by Wilson and Kirshner (1977), using perfused bovine adrenals, that the receptors in the cow medulla were entirely nicotinic. This was based on the inability of the muscarinic agonist pilocarpine to stimulate the release of CAs. However, in the bovine species, it appears that mimicking muscarinic stimulation of cultured medullary cells (with cyclic GMP analogues) can antagonize the nicotinic-induced release of CAs (Derome et al., 1981). Using a more physiological model, the perfused bovine adrenal, Swilem and coworkers (1983) showed similar results with muscarinic agonists inhibiting the nicotine-induced release of CA.

Paradoxically, in the studies by Wilson and Kirshner (1977) using perfused bovine adrenals, atropine, a muscarinic antagonist, was able to inhibit the nicotine-induced secretion of CA. However, the concentration needed for this effect (30 μM for half-maximal inhibition) was higher than that required for muscarinic blockade, suggesting that the effect of atropine was at the nicotinic receptor in these studies (Wilson and Kirshner, 1977). Therefore, in the bovine species, nicotinic-induced release of CAs seems to be negatively modulated by the muscarinic receptor of the adrenal medulla. However, this negative modulation does not appear to play a major role in the ACh-stimulated release of CAs, since the response of chromaffin cells to stimulation by nicotine is identical to the response evoked by stimulation with ACh (Derome et al., 1981). This therefore suggests that any muscarinic component of the ACh stimulation is overcome by the nicotinic-induced release of CAs.

In contrast, in human adrenal medullary cell pieces, ACh-evoked depolarization and release of ATP (reflecting secretion of chromaffin granule contents) is under both nicotinic and muscarinic control (Nassar-Gentina et al., 1990). In addition, in cultured guinea pig chromaffin cells, both nicotinic and muscarinic activation induced CA secretion, neither of which alone were as effective as ACh (Role and Perlman, 1983). Co-treatment of cells with optimal concentrations of nicotinic and muscarinic agonists induced a CA secretion comparable to secretion in response to optimal concentrations of ACh (Role and Perlman, 1983). In the mouse, adrenal medullary cells depolarize in response to muscarinic but not nicotinic stimulation (Nassar-Gentina et al., 1988), and can be blocked by atropine (100 μM), suggesting muscarinic receptor-mediated regulation of CA secretion in the mouse (Nassar-Gentina et al., 1990). In the perfused rat adrenal, both nicotinic and muscarinic stimulation of CA secretion have been observed, effects blocked by their respective antagonists, hexamethonium and atropine (Wakade and Wakade, 1983).

Therefore, in most other species studied, both muscarinic and nicotinic receptors seem to be involved in the release of adrenal medullary CAs. In the bovine species, CA release is stimulated by nicotinic receptor activation, this release perhaps being modulated by muscarinic receptor activation. However, ACh and other neurotransmitters are released from the splanchnic nerve, as discussed in previous sections. Therefore, receptors for many of the neurotransmitters identified in the splanchnic nerve have been investigated for their presence on chromaffin cells, and are discussed below.

### I.5.2.6 Receptor types on chromaffin cells

Regulation of CA secretion by multiple factors is achieved via receptors localized on chromaffin cell membranes. The presence of a number of receptors for a variety of neurotransmitters and neuromodulators has been suggested by biochemical and/or pharmacological experiments. These include cholinergic receptors of both the muscarinic (Kayaalp and Neff, 1979) and nicotinic (Higgins and Berg, 1987; 1988; Criado et al. , 1992; Lee et al., 1992) subtypes, opiate receptors (Chavkin et al., 1979; Kumukara et al., 1980; Lemaire et al., 1981; Lemaire et al., 1983), substance P receptors (Livett et al., 1979; Boksa et al., 1982), and prostaglandin receptors (Helle and Serck-Hanssen, 1975; Karaplis et al., 1989). In addition, functional and/or ligand binding studies in the adrenal medulla have suggested the presence of receptors for catecholamines, such as dopamine receptors (Artalejo et al., 1985; Quik et al., 1987), and both alpha and beta subtypes of adrenergic receptors (Greenberg and Zinder, 1982; Wada et al., 1982; Sakurai et al., 1983; Michener and Peach, 1984; Powis and Baker, 1986; Sharma et al., 1986), although the presence or functional role for the adrenergic receptors remains controversial (Wan et al., 1988). Finally, receptors for corticotropin releasing factor (CRF; Udelsman et al., 1986), vasoactive intestinal peptide (VIP; Wilson, 1988), adrenocorticotropic hormone (ACTH; Michener et al., 1985), histamine (Marley et al., 1991), imidazolines (Regunathan et al., 1990; Regunathan et al., 1991), arginine vasopressin (AVP) and

oxytocin (Taylor et al., 1989), and atrial natriuretic peptide (ANP; Heisler and Morrier, 1988) have been reported. Therefore, the multitude of receptors localized to chromaffin cell membranes for the variety of peptides and hormones may play a role in the regulation of secretion of chromaffin cell contents. In addition, any or all of these receptors for splanchnic nerve contents may be involved in the regulation of CA biosynthetic enzymes such as TH and PNMT.

### I.5.2.7 Neural regulation of TH and PNMT

#### TH

Evidence for neural regulation of adrenal medullary TH has been reported by a number of groups. Several studies have shown an induction of TH activity and/or mRNA by short-term drug treatment (Patrick and Kirshner, 1971; Zigmond et al., 1989; Stachowiak et al., 1990a; McMahon and Sabban, 1992). Reflex increases in splanchnic neuronal firing following 6-hydroxydopamine administration significantly elevated the activity of TH (Mueller et al., 1969a; Thoenen et al., 1970). In addition, increasing sympathetic neuron activity in vivo by reserpine administration elevated TH activity and/or mRNA (Mueller et al., 1969b; Molinoff et al., 1970; Black et al., 1971; Schalling et al., 1991). Abolishing neuron activity by splanchnic denervation resulted in decreases in TH mRNA (Schalling et al., 1991). These in vivo studies suggested that the nerve plays an important role in the regulation of TH.

As well, in vitro studies with bovine adrenal medullary cells have shown that continuous (6-48h) exposure to cholinergic nicotinic agonists increases TH mRNA (Stachowiak et al., 1990a; Craviso et al., 1992). However, in vivo ganglionic blockade by chlorisondamine did not reverse the reserpine-induced increase in TH mRNA (Schalling et al., 1991). This suggests that an additional neural transmitter not acting on nicotinic cholinergic receptors may be involved in regulation of TH expression. However, chlorisondamine, at the doses used (10 mg/kg ip), may not fully block the

adrenal nicotinic receptors. The general autonomic blockade evoked by this agent may result in a secondary increase in transmission at synapses on chromaffin cells using a messenger that is not blocked by this drug (Schalling et al., 1991). In support of this, regulation of TH activity has been observed in chromaffin cells or PC12 cells in response to VIP, secretin, nerve growth factor, epidermal growth factor, and glucagon (see Zigmond et al., 1989). These neurotransmitters/neuromodulators may also regulate the expression of the TH gene, and may account for the in vivo increase in TH mRNA following chlorisondamine treatment (Schalling et al., 1991), and the in vitro increase following elevation of intracellular cAMP levels (Carroll et al., 1991). Therefore, these studies support a neuronal regulation of TH activity and/or mRNA levels, mediated both by cholinergic receptor activation as well as by other splanchnic nerve contents.

**PNMT**

Although the predominant regulation of adrenomedullary PNMT activity is thought to be hormonal in nature (discussed in later sections), a number of studies have provided evidence for the neural regulation of this enzyme. For example, drug-induced increases in neural activity have been reported to induce PNMT (Mueller et al., 1969a; Molinoff et al., 1970; Thoenen et al., 1970; Ciaranello and Black, 1971; Ciaranello et al., 1976). 6-hydroxydopamine-induced increases in splanchnic neuronal firing significantly elevates levels of PNMT (Mueller et al., 1969a; Thoenen et al., 1970), an effect independent of pituitary influence and dependent on adrenal innervation (Thoenen et al., 1970). In vivo, a decrease in PNMT mRNA has been reported following denervation of the splanchnic nerve (Schalling et al., 1991), suggesting that the presence of the nerve is important for maintaining steady-state levels of PNMT. In addition, in vivo reserpine administration, which causes a reflex increase in neuronal activity, elevates the activity of PNMT (Mueller et al., 1969b; Molinoff et al., 1970; Black et al., 1971; Ciaranello and Black, 1971; Ciaranello et al., 1976). On the other hand, reserpine decreases

adrenomedullary PNMT mRNA, both in vitro (Stachowiak et al., 1990a) and in vivo (Schalling et al., 1991). The effect of reserpine administration on in vivo PNMT mRNA levels can be blocked by previous splanchnic nerve transection or ganglionic blockade (Schalling et al., 1991). These opposing results of reserpine on the activity and mRNA of PNMT are difficult to reconcile. One possiblity that has been suggested is that in vivo, reserpine may change the sensitivity of chromaffin cells to GCs (Dagerlind et al., 1990). Therefore, there would be enough GCs to increase the activity of PNMT (by inhibiting degradation and stabilizing the cofactor for this reaction; Ciaranello, 1978; Berenbeim et al., 1979; Wong et al., 1985), but not enough to stimulate the synthesis of new PNMT molecules. This theory does not however explain the results obtained in vitro, for which those authors (Stachowiak et al., 1990a) do not provide an explanation. Nevertheless, the studies described above illustrate that PNMT can be regulated by altering splanchnic neuronal tone, and that a neuronal cholinergic mechanism is likely responsible for this regulation.

The influence of a cholinergic mechanism involved in the regulation of PNMT is supported by in vitro studies showing that continuous (18h) exposure of bovine adrenal medullary cells to cholinergic nicotinic agonists increases PNMT mRNA (Evinger et al., 1988; Stachowiak et al., 1990a). In vitro, an increase in the activity and/or mRNA of PNMT in bovine adrenal medullary cells has also been reported following nerve growth factor (NGF) treatment (Acheson et al., 1984), histamine (Evinger et al., 1988), angiotensin (Stachowiak et al., 1990b), and imidazole receptor activation by the agonist, clonidine (Evinger et al., 1989). In addition, PNMT mRNA has been shown to be increased in vitro by elevating intracellular cAMP levels (Carroll et al., 1991), a second messenger used by a number of splanchnic nerve contents. Therefore, the influence of a number of splanchnic nerve contents, in addition to ACh, is apparent in the regulation of adrenomedullary PNMT.

Adrenomedullary PNMT can be regulated by splanchnic nerve contents, as discussed above. However, the predominant regulation of this enzyme is via the GCs released from the adrenal cortex, both under basal conditions, and during a stress response (see later sections). The reason for this relationship between the adrenocortical GCs and the adrenomedullary CAs is due to the anatomical association of the adrenal cortex and medulla. The medulla is exposed to levels of GCs in the order of $10^{-6}$ M or higher in both anesthetized (Kitay, 1961; Peytremann et al., 1973; Jones et al.,1977) and awake animals (Engeland et al., 1989), probably rising to $10^{-5}$ - $10^{-4}$ M during stress (Jones et al., 1977; Engeland et al., 1989). The concentrated levels of GCs bathing adrenal medullary cells will be diluted upon entering the general circulation. Therefore, tissues other than the adrenal medulla are exposed to GC concentrations found in the general circulation, in the range of $10^{-8}$ - $10^{-7}$ M, rising to $10^{-6}$ M during stress (Zumoff et al., 1974; Schoneshofer and Wagner, 1977; Dallman et al., 1987). This 100-fold discrepancy between the medulla and other tissues is due to the unique association of the cortical and medullary blood supply.

### I.5.2.8 Blood supply

Arterial blood to the adrenal cortex is supplied by the middle and inferior suprarenal arteries as well as the descending aorta. Blood is returned to the inferior vena cava via the suprarenal veins. Running through the center of the adrenal gland, at the corticomedullary border, is the central vein, draining blood from the cortex and medulla. Cortical blood, rich in corticosteroids, passes into medullary sinusoids. Therefore, it has been hypothesized that the CA synthesized by a given chromaffin cell depends on the concentration of hormones present in the cell's environment (Coupland and MacDougall, 1966; Pohorecky and Wurtman, 1971).

This view has been challenged by Coupland and Selby (1976) who, using an injected dye in order to visualize vessels with light microscopy, have shown that both A-

and NA-storing cells are found in proximity to cortical venous channels. Using epoxy resin-embedded sections and transmission electron microscopy, Kikuta and Murakami (1984) supported the work of Coupland and Selby (1976), and suggested that both types of chromaffin cells (A and NA) may have equal access to cortical hormones. Both A and NA cells were found in proximity to all types of blood vessels and did not follow the selective medullary distribution of the venous channels (draining cortical blood flow) and medullary capillaries (containing arterial blood transmitted via the medullary arteries).

However, Livett and coworkers (1982) have shown that A-storing cells are located preferentially towards the cortico-medullary border, whereas the NA-storing cells are found in the vicinity of the central lobular vein. In addition, the outer margin of the adrenal medulla expresses mRNA for PNMT in ovine adrenals as early as 95d gestation (Wan et al., 1989a), and in adult bovine and ovine adrenals, colocalizing with proENK mRNA (Wan et al., 1989b). Taken together, these studies raise the point that an adult NA-storing cell found in the vicinity of cortical venous effluent may retain the NA phenotype even in the presence of high GCs. This suggests that since adult medullary NA cells do not contain PNMT, they may not be subject to regulation by GCs. However, embryonic rat adrenal medullary precursor cells in vitro can differentiate from a NA phenotype to an A phenotype by the addition of GCs (Seidl and Unsicker, 1989). Therefore, it appears that developmental determination of the NA vs. A phenotype in chromaffin cells may be influenced by GC exposure (see also section I.6.2). However, in the adult, GC regulation of CA biosynthetic enzymes (e.g. PNMT) and of peptide levels only occurs in susceptible (e.g. PNMT- and A-containing) sub-types of chromaffin cells.

The previous sections have dealt with the biosynthesis of CAs, as well as the composition of both adrenal medullary cells and the splanchnic nerve innervating these cells. In addition, control over the secretion of CAs, as well as the neural regulation of

CA synthesis have been examined. The functional interaction of the adrenal cortex and medulla is brought about by the unique juxtaposition of these two tissue as well as by the adrenal blood supply. The supply of blood to the adrenal medulla can explain why chromaffin cells are exposed to such high levels of GCs, which results in a regulation of CA biosynthetic enzymes. In order to understand the nature of GC regulation of adrenal medullary function, the next sections will deal with the mechanism and regulation of the adrenocortical synthesis of GCs. In addition, the interaction between adrenocortical GCs and adrenomedullary function during development and adulthood will be examined in greater detail.

### I.5.3 The adrenal cortex

#### I.5.3.1 Steroid hormone synthesis

Steroid hormones are structures made up of a cyclopentanophenanthrene nucleus, which is a cyclopentane group attached to a phenanthrene core, with adrenal corticosteroids existing in the "chair" formation. The precursor to all steroid hormones is cholesterol ($C_{27}H_{45}OH$), 80% of which is derived from serum cholesterol (Gwynne and Strauss, 1982). The rate limiting step in adrenocortical hormone biosynthesis is the conversion of cholesterol to pregnenolone (Simpson and Waterman, 1983).

#### I.5.3.2 Regulation of GC synthesis by ACTH

The regulation of adrenal steroid production is under the control of a neuroendocrine axis made up of the hypothalamus, pituitary and adrenal gland (HPA axis). The hypothalamic peptides, corticotropin-releasing factor (CRF) and arginine vasopressin (AVP), trigger the synthesis and release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. Adrenal corticosteroids are released in response to stimulation by ACTH within 2 to 3 min of exposure (Sydnor and Sayers, 1954). Both low and high affinity receptors for ACTH are found on cortical cells (Lefkowitz et al.,

1971; McIlhinney and Schulster, 1975). The high affinity receptor is thought to be responsible for the first phase of adrenal response to ACTH, which occurs within the first 5 min after an intraperitoneal injection of ACTH (Cam and Bassset, 1983). This phase is due to release of stored corticosteroids (Cam and Bassset, 1983), and includes increased rate of steroid synthesis (Cam and Bassett, 1986). An increase in steroid synthesis involves mobilization of cholesterol stored as esters in the adrenal gland (Brown et al., 1979; Gwynne and Strauss, 1982). The second, much slower phase (≈20 min later) results in an ACTH dose-dependent increase in steroidogenesis (Cam and Bassset, 1983; 1986). Therefore, both release and synthesis of GCs occur in response to adrenocortical activation by ACTH.

### I.5.3.3 Negative feedback

The pattern of release of corticosteroids into the bloodstream depends on both environmental factors (stressors) and on the endogenous daily (circadian) rhythm of secretion. In the rat, the circadian peak occurs during the most active phase of the daily cycle (in the dark phase, during the feeding cycle). GCs participate in a negative feedback regulation of ACTH by inhibiting the release of both CRF and ACTH at the level of the hypothalamus and pituitary, as well as at higher brain centers. Depending on the timing of exposure of the organism to B, there can be three types of feedback control over ACTH secretion: fast feedback (seconds to minutes), intermediate feedback (2-10h) and slow feedback (hours to days) (for reviews see Keller-Wood and Dallman, 1984; Dallman et al., 1987). This efficient control over adrenal GC secretion following a stressor occurs in order to prevent detrimental effects of prolonged GC exposure, as discussed in earlier sections.

The secretion of GCs from the cortex occurs in response to ACTH during resting states and stressful stimuli. During stress, both GCs and neural influence regulate adrenal CA synthesis (see above). However, it is unclear whether neural regulation of

CA biosynthetic enzymes is due to direct neural influence on the adrenal medulla solely, or whether the adrenal cortex plays an indirect role in this process, since the autonomic greater splanchnic nerve innervates both the adrenal medulla and cortex.

### I.5.3.4 Innervation of the adrenal cortex

Although the splanchnic nerve is thought to primarily terminate on the chromaffin cells of the medulla, a small proportion of filaments innervate adrenocortical arteries, arterioles and veins (Allaben, 1982). Nervous input to the cortex appears to influence adrenocortical function since splanchnic nerve stimulation has been shown to release cortisol from perfused porcine adrenals (Bornstein et al., 1990). Perfusion of rat adrenals with both ACh and carbachol (a predominantly muscarinic agonist) caused a release of GCs, presumably by acting on cholinergic muscarinic receptors (Porter et al., 1988). In addition, cholinergic stimulation of bovine adrenocortical cells in culture has been reported, with the muscarinic receptor involved in this stimulation (Hadjian et al., 1982). In those cells, acetylcholine was found to be steroidogenic, having a synergistic effect in combination with ACTH (the hormonal signal for steroidogenesis). Therefore, regulation of CA biosynthetic enzymes by neuronal influences, as suggested by splanchnic denervation and pharmacological studies, may be either due to a direct effect of the nerves on the chromaffin cells, or due to an indirect potentiation of steroidogenesis, increasing the exposure of the medulla to GCs.

Using perfused porcine adrenals with an intact nerve supply, Bornstein and coworkers (1990) have shown an increase in adrenocortical steroid secretion following perfusion with A and NA. Although these authors mention the possibility that the observed effect is due to CAs originating in adrenocortical catecholaminergic fibers (presumably NA), they postulate that in vivo, CAs may be released from medullary cells and act in a paracrine fashion. Therefore, in acute stress situations, released CAs could conceivably increase the synthesis of GCs. The increase in GC release could in turn

enhance the synthesis of CAs, which would ensure that any CA lost during the stressor could be replenished by the GC actions on chromaffin cell biosynthetic enzymes. This putative short feedback loop could thus regulate steroidogenesis via a neuroadrenocortical axis (Bornstein et al., 1990). Although this putative neuroadrenocortical interaction is the result of the unique association of these two tissues, it is not well understood. Studies in the past have concentrated on the well-known interaction of GCs and CA synthesis. It is important here to describe this interaction at the level of both development and adulthood, in order to understand the relevance of studying the dynamics of such an interaction.

## I.6 Relationship between GCs and CAs

### I.6.1 Development of the adrenal medulla

The adrenocortical environment has been suggested to be important for the development of adrenal medullary cells in mammalian species, where the main catecholamine is A (Coupland, 1953). The adrenal medulla is part of the sympathetic nervous system and is derived from the ectodermal neural crest. Precursor cells originate from the thoracolumbar portion of the developing neural crest (Hammond and Yntema, 1947; LeDouarin and Teillet, 1974; LeDouarin, 1984), migrate and are encapsulated by the adrenal cortex at embryonic days 12 to 15 (E12-E15) in the rat (Bohn et al., 1981; Millar and Unsicker, 1981). Local environmental signals determine the pathway of differentiation of neural crest cells into either chromaffin cells or into sympathetic neurons (Anderson and Michelson, 1989). GCs secreted from the adrenal cortex are a likely environmental factor important for the development of chromaffin cells (Anderson and Michelson, 1989) and particularly for the appearence of the adrenergic phenotype (Milkovic et al., 1971; Cohen, 1976; Bohn et al., 1981; Teitelmann et al., 1982). The embryonic development of adrenal PNMT occurs at E16.5 to 17.5, several days after progenitor cells have chosen the chromaffin cell pathway of development (Bohn et al.,

1981; Teitelmann et al., 1982). Adrenal PNMT reaches adult levels during the late fetal period (Verhofstad et al., 1985), with adult steady-state levels of PNMT mRNA reached prior to E18 (Ehrlich et al., 1989).

### I.6.2 GCs and the development of PNMT

GCs are known to regulate adult levels of PNMT (Wurtman and Axelrod, 1965), and were initially thought to control the appearance of adrenal PNMT during development. Rat embryonic adrenal steroidogensis can be detected as early as E12.5 (Roos, 1967) whereas the appearance of PNMT occurs at E16.5-17.5 (see above). The mRNA for the GC receptor appears one full day's gestation prior to the appearance of the mRNA for PNMT (E15.5 vs. E16.5) (Anderson and Michelson, 1989). However, the GC receptor cannot be detected prior to E17.5 (Seidl and Unsicker, 1989), suggesting that the presence of the receptor, and therefore GCs, are not necessary to trigger the induction of PNMT during development. In support of this notion, fetal adrenal glands maintained in organ culture from day E16 onwards, in the absence of GCs, will still express PNMT activity at the same developmental period as in vivo (Teitelmann et al., 1982). In addition, the timing of appearance of PNMT immunoreactive cells is not affected by modulating GC concentrations, since high doses of GCs given to a pregnant rat, or GCs or ACTH given directly to the embryos via a transuterine route, did not trigger the premature expression of PNMT in the embryos (Bohn et al., 1981). These studies suggested that the presence of GCs was not necessary for the induction of PNMT. However in the in vivo study, the injected GCs may have inhibited endogenous secretion, thus preventing an increase in PNMT. Additionally, the ability of embryonic ACTH injections to elevate GCs in the adrenals or plasma was not investigated. In the organ culture study, the expression of PNMT observed could have been due to the small amount of GCs present in the adrenal cortex. In vitro, it has been demonstrated that precursor cells which do not contain PNMT activity or A levels can be made to do so by

the addition of DEX to the culture medium (Seidl and Unsicker, 1989). Therefore, it remains possible that GCs are the trigger for the expression of adrenomedullary PNMT, and that during embryogenesis, a threshold level of GC receptors may be required to mediate the induction of PNMT by GCs (Anderson and Michelson, 1989).

Although it is still unresolved whether the initial induction of PNMT is triggered by GCs, these steroids are critical for the normal ontogenic increase in PNMT activity (Bohn, 1983). Normal increases in fetal PNMT activity are prevented by embryonic hypophysectomy prior to E17.5 (Margolis et al., 1966; Bohn et al., 1981) and enhanced by GC treatment of pregnant rats during late gestation (Bohn et al., 1981). Additionally, the regulation of PNMT activity later in development is subject to neural influence due to subsequent innervation of chromaffin cells by the splanchnic nerve (Bohn, 1983). During embryonic development, Verhofstad et al. (1985) using immunohistochemistry and biochemistry have shown the presence of a population of chromaffin cells that contain both A and NA. These studies showed that the phenotype of *either* A *or* NA granule storage does not occur until post-natal days 2 to 4 in the rat.

Therefore, the initial expression, subsequent maturation and modulation of the adrenergic phenotype is subject to a number of different environmental and/or genetic factors. What is clear is that GCs play an important and crucial role in the developmental phenotype of chromaffin cells. In addition, during adulthood, GCs play a role both in the maintenance of adrenal CA biosynthesis and the induction of this synthesis during times of stress. Therefore, the next section will describe what is known about the effect of GCs, in the adult, on the regulation of two CA biosynthetic enzymes, TH and PNMT, enzymes which have been studied in the present thesis. This will help the reader understand the reason for studying the dynamics of short-term exposure to GCs on the regulation of these enzymes.

### I.6.3 GC regulation of TH and PNMT in adults

#### TH

TH is regulated both by neural (see previous sections) and hormonal changes which occur during acute and chronic stressors (Kvetnansky et al., 1970; Kvetnansky et al., 1971; Kvetnansky, 1973; Thoenen et al., 1973; Guidotti et al., 1973; Otten et al., 1973; Hoffman et al., 1975; Bhagat and Horenstein, 1976). As discussed in section I.5.2.7, there are studies which suggest that stress-induced regulation of TH is neurally-mediated. However, there are also studies in the literature which support a role for GCs in the regulation of this enzyme. For example, hypophysectomy has been shown to reduce the activity of TH, with restoration by ACTH administration (Mueller et al., 1970). Chronic (14d) DEX treatment [at a concentration that would suppress endogenous corticosterone (B)] decreased the activity of TH (Mitchell and Vulliet, 1985). In addition, adrenal medullary TH mRNA was reduced following hypophysectomy and reversed by GC administration (Stachowiak et al., 1988; Jiang et al., 1989). Mueller et al. (1970) have suggested that ACTH is required to maintain basal levels of TH but that in order to elevate TH levels above control values, increases in nerve impulses are required. However, GC-induced increases in TH mRNA levels above control values have been observed in adrenal medullary cells in culture (Stachowiak et al., 1990a) and in cells from the adrenal medullary tumor cell line PC12 (McMahon and Sabban, 1992). Therefore, taken together, these studies support the ability of GCs to regulate TH.

#### PNMT

Regulation of PNMT activity by GCs was reported in the mid 1960's. Early studies suggested that GCs play a role in the long-term maintenance of steady state PNMT levels (Wurtman and Axelrod, 1966; Fuller and Hunt, 1967; Thoenen, et al., 1970; Ciaranello and Black, 1971). This was largely based on the observation that hypophysectomy reduced adrenomedullary PNMT activity, with restoration of this activity by administration of ACTH and/or GCs (Wurtman and Axelrod, 1965, 1966).

The fall in PNMT activity after hypophysectomy was reported to be caused by a decrease in the number of enzyme molecules, as measured by immunotitration studies (Ciaranello, 1978). Further evidence for GC regulation of PNMT came from elegant studies in which rat adrenomedullary explants were grown for 21d in the anterior chamber of the eye (Pohorecky et al., 1969). This allowed elimination of the influence of adrenal innervation, in order to observe the effect of GC treatment. Medullary explants, taken from the left adrenal gland, had approximately 2% of the amount of PNMT activity found in the medulla of the intact right adrenal gland. 7 day DEX treatment (1 mg/kg) caused a 10-fold increase in the activity of PNMT in the explant, without changing activity in the intact adrenal. Systemic injection of 1 mg/kg DEX will increase the concentration of GCs bathing the explanted medulla, thus exposing it to very high concentrations of GCs. However, this DEX treatment is unlikely to affect basal levels of PNMT in the intact adrenal since the GC concentration in the adrenal medulla, already at high levels, cannot be raised further by this injection. This dose of injected DEX would inhibit endogenous GC secretion, thus decreasing the level of GCs reaching the intact adrenal medulla. Nevertheless, these studies clearly show an ability of GCs to increase PNMT activity, and suggest that chromaffin cells do not need concurrent neural inputs for GCs to increase the activity of PNMT.

Subsequent studies showed that elevation in GC secretion may play a role in increasing PNMT activity on a long-term basis. For example, in vivo increases in adrenal PNMT activity were found following chronic stress (Kvetnansky et al., 1970), prolonged ACTH administration (Vernikos-Danellis et al., 1968; Simonyi et al., 1985), or mother-infant separation (Breese et al., 1973). In vitro, continuous (18h-2d) exposure of adrenal medullary cells to GCs increased the activity of PNMT (Hersey and DiStefano, 1979; Kelner and Pollard, 1985). However, this increase was not apparent earlier, suggesting that regulation of PNMT by GCs occurred by a process that required prolonged exposure to the steroid. Following a hypophysectomy-induced decrease in PNMT enzyme

molecules, GCs were shown to increase the amount of immunotitratable PNMT molecules (Ciaranello, 1978). In addition, earlier studies by Ciaranello and Black (1971) had suggested that GCs increase the rate of enzyme synthesis in hypophysectomized rats. More recent studies have shown that GCs can increase the levels of the mRNA for PNMT (Stachowiak et al., 1988; Wan and Livett, 1989; Stachowiak et al., 1990a), suggesting that GCs regulate PNMT by increasing the amount of enzyme molecules. In addition, using a double labelling technique to measure relative rates of enzyme degradation and synthesis, Ciaranello (1978) illustrated that GCs increased the amount of enzyme molecules by decreasing the rate of PNMT degradation. The action of GCs on the stability of PNMT molecules was thought to be through regulation of the "endogenous stabilizing factor" S-adenosyl methionine (SAM), the methyl donor for PNMT (Berenbeim et al., 1979; Wong et al., 1985). Therefore, the regulation of PNMT activity by GCs appears to be both via a stabilization of SAM and by increasing the amount of protein syntheis.

Studies suggest that the regulation of PNMT activity by GCs requires long-term increases in GC release, such as during chronic stress, as mentioned above. However, there are a few in vivo studies which illustrate an increase in PNMT activity following acute manipulations such as stress. In situations involving relatively short (1-2.5h) but severe stressors, such as cold, forced swimming or immobilization, small increases in adrenal PNMT activity have been observed (Kvetnansky et al., 1970; Kvetnansky, 1973; Hoffman et al., 1975; Bhagat and Horenstein, 1976). The increase in PNMT activity is apparent 6-32h post-stress and is abolished by hypophysectomy (Kvetnansky, 1973; Bhagat and Horenstein, 1976). However, in all of these studies, only one time point following the stressor was chosen to measure the activity of PNMT. No study has systematically investigated the effect of a single episode of stress on PNMT activity at various time points following this stressor. Since this idea has not been systematically studied, we sought to examine the possibility that PNMT could be regulated by very short

increases in the levels of GCs, similar to what occurs during short-term mild stressors. We are reporting that indeed PNMT can be increased by exposure of cultured medullary cells to very short pulses of GCs, as long as the enzyme is measured following a time delay. The present thesis is the first example of such a demonstration, and extends these initial findings by examining the dynamics of the receptor involved in this regulation, the GC receptor. The cultured medullary cells afforded us an ideal model in which to study this regulation since the levels of GCs could easily be controlled. In addition, the timing of exposure of cells to GCs could be regulated, thus allowing us the opportunity to dissect out the time constraints involved in the regulation of PNMT by hormonal influences, findings which we have also extended to the in vivo situation.

Hormonal influence over the regulation of PNMT activity seems to be due to the unique association of the medulla to the cortical surrounding tissue, and thus to GCs. However, there are other hormones or peptides released during a stress response which may be involved in the regulation of this enzyme. This may include circulating hypothalamic CRF or pituitary ACTH or opioids, since receptors for these peptides have been localized on chromaffin cell membranes (see previous sections). To date, however, it is unclear what the role of these peptide receptors are in the adrenal medulla, and whether the circulating levels of these neurotransmitters are high enough to affect the regulation of adrenomedullary PNMT.

Other adrenocortical hormones such as the mineralocorticoids, the androgens or the estrogens, which may be secreted in high enough levels to affect the medullary enzymes, may be able to regulate PNMT. Some of these steroids, such as aldosterone, cortexolone (both of which are mineralocorticoids) and 11ß-hydroxyprogesterone were able to partially prevent a loss in the activity of PNMT in medullary cells cultured for 5 days (Hersey and DiStefano, 1979). This loss was preventable by the addition of the GCs, DEX, cortisol or corticosterone. However, the presence of receptors for steroids other than GCs has not been studied to date. In the present thesis, we have reported that

we were unable to measure the presence of mineralocorticoid receptors using ligand binding techniques. However, there does exist the possiblity that these and other steroid receptors may be present in adrenal medullary cells and may play a role in the regulation of PNMT.

### I.6.4 GCs and the expression of adrenomedullary ENK levels

During a stress response, the GCs released from the adrenal cortex regulate chromaffin cell levels of both CAs and ENKS. [However, to our knowledge, GC regulation of the remainder of the adrenomedullary peptides (in Table I-1) has not been studied to date (see Winkler et al., 1992).] GCs regulate adult levels of PNMT coordinately with chromaffin cell ENK levels. Naranjo et al. (1986) has provided evidence for a DEX-induced increase in mRNA for proENK (the precursor peptide for ENK) and in ENK immunoreactivity in bovine medullary cells cultured in low GC medium. In addition, LaGamma and Adler (1987) using rat adrenal medullary explants, reported concentration-related increases in Leucine (L)-ENK (as measured by radioimmunoassay) in response to B. Using a similar preparation with the exception that the rats were previously hypophysectomized to minimize endogenous GCs, Inturrisi et al. (1988) showed increases in ENK-containing peptides and preproENK mRNA following DEX treatment. In addition, medullary explants from non-hypophysectomized (sham-operated) rats treated with the GC receptor antagonist RU 38486 showed a reduction in the level of ENK-containing peptides, suggesting that GCs are important in rat adrenal ENK peptide biosynthesis (Inturrisi et al., 1988). In vivo, Stachowiak et al. (1988) has shown a decrease in adrenal medullary ENK levels following hypophysectomy, an effect that is reversed by GC administration. In vitro, using bovine adrenal medullary cells in culture, Stachowiak et al. (1990) have shown a coordinate regulation of mRNAs for both PNMT and ENK by DEX treatment, with maximal increases observed with $10^{-8}$ M

DEX. Therefore, these studies illustrate the ability of GCs to regulate not only the biosynthesis of CAs but also of ENKs.

The previous sections have examined the interaction between the GCs released from the adrenal cortex and adrenomedullary function. GCs have been shown to be involved in the developmental regulation of PNMT, as well as the regulation of adult levels of TH, PNMT, CAs and ENKs. However, the predominant notion is that GCs regulate PNMT and ENKs on a permissive or long-term basis. A more dynamic regulation of adrenomedullary function by GCs has not yet been clearly established. The GC regulation of adrenomedullary function, be it steady-state and/or dynamic, occurs via activation of GC receptors. Therefore, the following sections will examine in detail the dynamics, structure, cellular localization, and regulation of the GC receptor. In addition, the data published on the adrenomedullary GC receptor up until the inception of the present thesis will be reviewed. The present thesis markedly extends this field of research with a detailed look at the dynamics of the adrenomedullary GC receptor, the regulation of this receptor and the functional consequences of altering receptor levels on GC regulation of PNMT activity.

## I.7 The GC receptor

In the early 1970's, the presence of a cytosolic protein was discovered in the liver which bound to natural as well as to the synthetic GC, DEX (Beato and Feigelson, 1972). This protein demonstrated specific high affinity binding for both natural and synthetic GCs, and sedimented at 7S in a sucrose density gradient. This DEX-binding protein was proposed as the hepatic GC receptor (Beato and Feigelson, 1972). Using rat hepatoma (HTC) cells in culture, Baxter et al. (1972) have shown that when nuclei from these cells are incubated with [$^3$H]-DEX, no specific binding is observed. However, when the ligand is preincubated with cytosol and then incubated with the nuclei, specific

binding can be measured. This suggested that cytoplasmic receptors are needed for GC binding to nuclear sites in the steroid-responsive rat liver cells. In addition, since this nuclear binding was destroyed by DNase, and since cytosol-bound [$^3$H]-DEX bound to purified HTC cell DNA, the authors suggested that DNA is an obligatory component of the nuclear binding sites and therefore the site of action of the steroid-receptor complex.

### I.7.1 Consequences of steroid hormone binding

The current model (see Figure I-2) for the general mechanism of steroid hormone action at DNA sites (for reviews see Pratt et al., 1989; Landers and Spelsberg, 1992) incorporates the early idea that hormones control the production of "templates composed of nucleic acids" (Mueller et al., 1958). The current model is a more complete version of the one proposed by Jensen and coworkers (1968) and Gorski and coworkers (1968) almost 25 years ago for the estrogen receptor. Circulating steroids, not bound to plasma binding proteins, diffuse passively across target cell membranes due to their lipophilic nature. Once inside a target cell, steroids bind reversibly and with high affinity to intracellular receptors. The GC receptor has an apparent molecular weight of 300 kiloDaltons (kDa) and exists as an inactive oligomeric complex containing the 90-kDa heat shock protein Hsp 90 (Sanchez et al., 1985; Mendel et al., 1986b) as well as Hsp 56 (Sanchez, 1990) and Hsp 70 (Estes et al., 1987; Sanchez et al., 1990). The binding of hormone to receptor causes a change in the structure of the receptor, termed "activation" or "transformation", a process which releases the heat shock proteins and occurs within minutes of exposure to the steroid (Landers and Spelsberg, 1992). In the case of the GC receptor, the hormone-receptor complex is then translocated to the nucleus following hormone binding (Wikstrom et al., 1987; Picard and Yamamoto, 1987). The possible mechanisms accounting for the translocation of the hormone-bound GC receptor are described in greater detail in later sections.

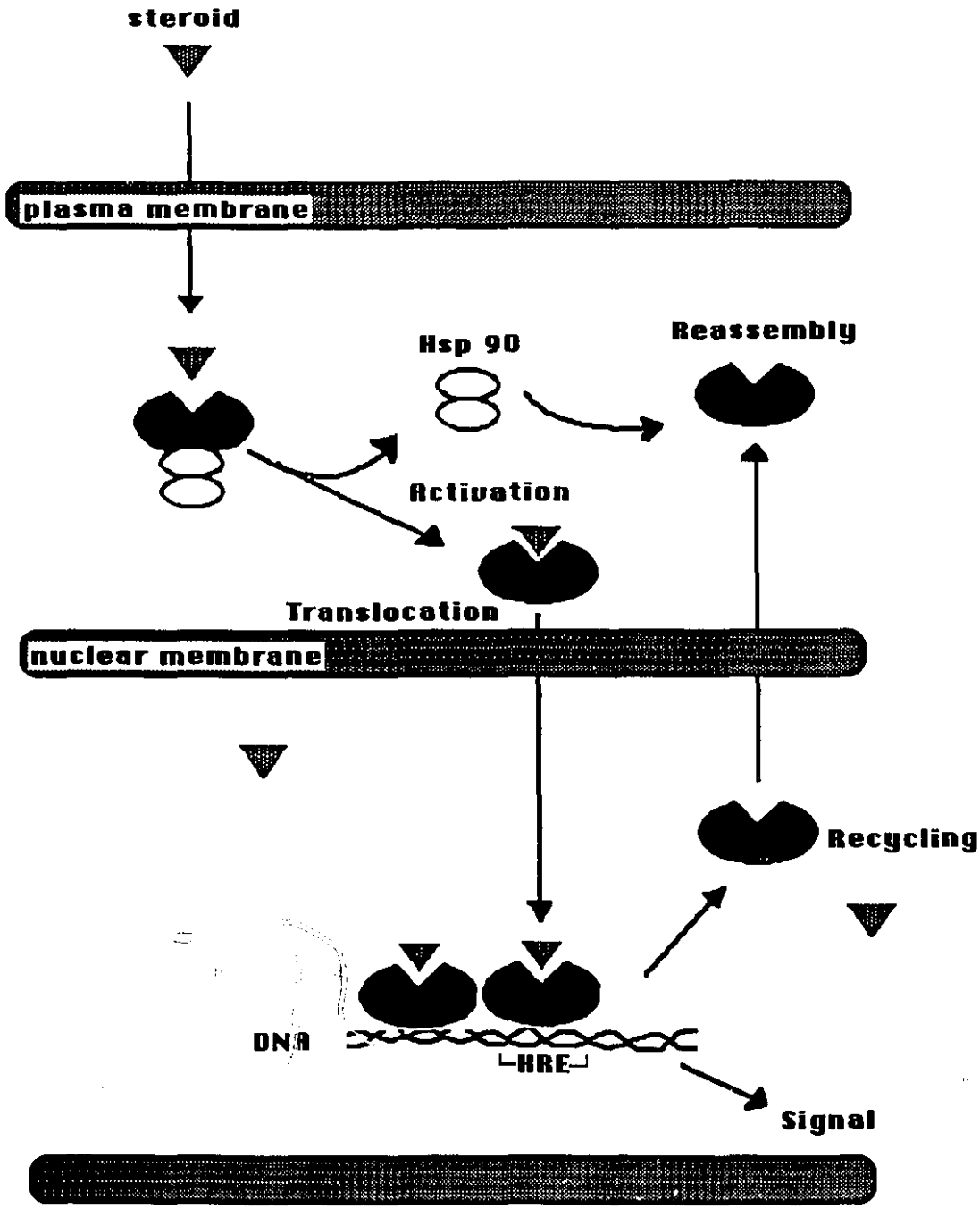


**Figure I-2: General mechanisms of steroid hormone action.** Steroids passively enter the cell and bind to soluble steroid receptors, causing the release of heat shock proteins (Hsp 90, 70 & 56 in the case of the GC receptor). This is termed activation. Once activated, the steroid-receptor complex, if present in the cytoplasm (as is the case for the GC receptor), translocates to the nucleus and binds to hormone response elements (HREs) on target DNA, possibly as a dimer of steroid receptor complexes. DNA binding leads to either repression or activation of target genes. The steroid receptor is then either broken down, or recycled and reassembled with heat shock proteins.

The binding of hormones to their receptors allows for binding of the hormone-receptor complex to DNA-acceptor sites, also known as hormone response elements (HREs), within 2 to 5 minutes following steroid exposure. The transformation of the ≈8-10S GC receptor to the DNA-binding state results in a receptor of reduced apparent molecular weight (94-100 kDa) with a sedimentation of 4S on sucrose density gradients (Pratt et al., 1989). Following hormone treatment of $GH_1$ cells, a decrease in the 10S receptor is accompanied by an increase in the 4S cytosolic and in nuclear-bound receptors (Raaka and Samuels, 1983). It is the 4S and not the 10S form that was shown to bind to purified DNA, suggesting that the 4S receptor is the form that exhibits nuclear binding (Raaka and Samuels, 1983).

Nuclear binding of the GC receptor occurs via a recognition sequence for this receptor on target DNA. Target genes can be either positively or negatively regulated by the hormone receptor complex, with negative regulation probably involving the inhibition of binding of an essential transcription factor to an element neighbouring the HRE (Landers and Spelsberg, 1992). The HREs are usually found as inverted repeats, making this pallindromic element ideal for binding of receptor homodimers. The GC receptor appears to bind to its HRE as a receptor dimer. The specificity of binding of the GC receptor to its HRE is increased at high salt conditions (570 mM), and can be described by a two-site cooperative (dimer) binding model (Härd et al., 1990). Dimerization most likely occurs prior to DNA binding and is necessary for a more efficient interaction of the receptor with its HRE (Cairns et al., 1991). The DNA sequence for the GC HRE has been identified (Evans, 1988), and has been found in the promotor region of the adrenomedullary PNMT gene (Ross et al., 1990), thus supporting a direct regulation of this enzyme by GCs.

The effect of steroids on target gene RNA or protein synthesis occurs over several hours. In general, the physiological effects of steroid receptor binding in any given target cell are observed within 12 to 24h following steroid treatment (Landers and Spelsberg,

1992), a time course supported by studies reported in the present thesis. Once GC receptors have exerted their actions at the level of the genome, it has been proposed that these receptors undergo an energy-dependent recycling process (Munck et al., 1972; Raaka and Samuels, 1983; Munck and Holbrook, 1984). Following removal of GCs from the media of cultures of $GH_1$ cells, there is a decrease in the levels of 4S and nuclear-bound GC receptors and a concomitant increase in the levels of 10S cytosolic receptors (Raaka and Samuels, 1983). Using dense amino acid labelling techniques, these authors showed that the increase in the 10S receptor was not due to synthesis of new receptors, suggesting that the receptors had been recycled from nuclear-bound receptors. In addition, in $GH_1$ cells, the recycling process of the nuclear-bound 4S receptor to the cytosolic 10S receptor was found to occur within 3h following removal of GCs, a time frame similar to that observed in the present observations of the dynamics of the adrenomedullary GC receptors. Therefore, there appears to be a cycle (see Figure I-2) involving binding of the hormone to its receptor, thus releasing the heat shock proteins and converting the receptor to the 4S form, followed by translocation and subsequent binding of this hormone-receptor complex to the DNA acceptor sites, and a recycling of these nuclear-bound receptors to a soluble pool of hormone-free receptor presumably reassociated with heat shock proteins (i.e. as a 10S form).

In studies which measure the levels of GC receptor using radioligand binding techniques, not all of the states of the receptor mentioned above can be accounted for. Preparation of a cytosolic fraction (which includes both cytoplasm and nucleoplasm) will yield all receptors which are soluble and not nuclear bound. The following states of the soluble receptor are obtained in the cytosolic (soluble) fraction. The first is receptors which are hormone-free and in a 10S form (i.e. associated with heat shock proteins). Radioligand studies allow measurement of these receptors with incubation times as short as 4h at 4°C under optimal binding conditions (Kalimi and Hubbard, 1983). The second is receptors already bound to endogenous hormone and free of the heat shock proteins

(i.e. a 4S form). These receptors can be measured under "exchange conditions", that is for 22h at 4°C, allowing the radioligand to exchange with endogenous hormone. The third pool of receptors is one that cannot be measured using radioligand binding techniques. This pool consists of receptors that are steroid-free but are not complexed with the heat shock proteins (i.e. a 4S form). This "transformed" receptor cannot bind ligand (since the receptor must be in a 10S form to bind steroid; Mendel et al., 1986a; Sanchez, 1992) and may represent the recycled pool of receptors. Proportions of soluble unbound (pool 1) and soluble bound (pool 2) receptors have previously been measured with ligand binding techniques (Reul and DeKloet, 1985; Meaney et al., 1988). However, we know of no studies which have compared levels of bound+unbound receptor pools to total levels of receptor, since the latter can only be measured with immunocytochemical techniques. Therefore, in the present thesis, as in any experiments using radioligand binding to measure GC receptors, we are only estimating the binding capacity of the cell and cannot account for the transformed pool of receptors. In addition, by preparing a cytosolic fraction, we are also losing any receptor that may have been nuclear bound. These technical limitations must be kept in mind when interpreting data using radioligand binding measurements as estimates of steroid hormone receptors.

Soluble receptor binding studies will allow estimates of receptors which are steroid-free or GC-bound, but not DNA-bound. A loss of soluble sites measured with radioligand binding following steroid treatment will provide an estimate of the translocation of this receptor to a DNA-bound pool. An additional method of studying this translocation is to measure nuclear binding of radioligand. The nuclear binding of the GC receptor occurs at 37°C and is stopped or prevented in vitro by placing the cells at 0°C (Raaka and Samuels, 1983). For the GC receptor, nuclear binding is assessed by measuring the levels of radiolabel in a prepared nuclear fraction, following exposure of cells to radioligand. This technique, in addition to the classical radioligand steroid binding assay, has been used in the present thesis to explore the dynamics of binding of

the adrenomedullary GC receptors following short-term exposure of medullary cells to GCs.

### I.7.2 GC receptor structure

The first structure of a steroid receptor was provided by studies which identified and cloned the GC receptor cDNA (Hollenberg et al., 1985; Meisfeld et al., 1986). The recent cloning and sequencing of the cDNAs for other steroid hormone receptors (estrogen, progesterone, androgen) has revealed homology between those receptors and receptors for thyroid hormones, retinoic acid, vitamin D as well as receptors for ligands as yet unknown (see Evans and Arriza, 1989). This led to the classification of steroid receptors within a superfamily of receptors that behave as ligand-dependent transcription factors (Evans, 1988). These receptors are characterized by the presence of a zinc finger DNA binding domain within their structures.

The structure of the GC receptor and all receptors in the steroid superfamily are composed of four separate domains: the N-terminal variable regulatory (immunogenic) domain, the DNA binding domain, a "hinge" domain, and a hormone-binding domain. The N-terminal domain is a variable region and is thought to be involved in receptor dimer formation as well as cell-type specific regulation of gene transcription (Landers and Spelsberg, 1992). This domain has been reported to be necessary for complete activity of the human GC receptor (Hollenberg et al., 1987), since cells transfected with GC receptors containing deletion mutations of this domain, reduces GC-mediated activity 3- to 20-fold.

The other three domains are highly conserved among the estrogen, progesterone and GC receptors. However, among the receptors from the superfamily, the most highly conserved region is the DNA binding domain. This domain forms two "zinc finger-like motifs" and is essential for the binding of the GC receptor to DNA (Hollenberg and Evans, 1988). In elegant experiments, Green and Chambon (1987) substituted the DNA-

binding domain of the estrogen receptor for that of the GC receptor, and demonstrated GC-induced function following estradiol treatment. These studies illustrated the importance of the DNA-binding domain for determining the specificity of target gene activation by hormones. Studies by Hollenberg and coworkers (1987), using deletion mutants of the GC receptor, saw no binding to DNA cellulose or GC-mediated activity of transfected receptors missing base pairs 428 to 490 (the DNA-binding domain). However, more recent studies have suggested that the presence of the HRE alone is not sufficient for hormone inducibility, but that other DNA regulatory elements, such as those for transcription factors, are required (Strähle et al., 1988).

Hollenberg and coworkers (1987) were the first to demonstrate that removal of the steroid binding domain resulted in a constitutively active GC receptor mutant. This led to the proposal that binding of the steroid hormone causes some sort of conformational change in the structure of the receptor protein such that the DNA binding domain is no longer inhibited by the steroid binding domain. This view of steroid receptor action was held for a number of years. However, subsequent studies have shown that it is the heat shock proteins which are released in response to ligand binding (see Landers and Spelberg, 1992) and not a physical unmasking of the DNA binding domain by the steroid binding domain. These and other studies took advantage of a model system utilized for GC receptor gene regulation - a plasmid containing the mouse mammary tumor virus coupled to the enzyme reporter gene chloramphenicol acetyltransferase (MMTV-CAT) and transfected into a eukaryotic cell line. This retrovirus can be induced by GCs in both mouse and rat cell systems (Ringold, 1979). Using this in vitro model, Willmann and Beato (1986) had demonstrated that heat-activated steroid-free GC receptors were able to bind to the HRE on MMTV. This binding was dependent on previous heat activation of the hormone-free cytosol, and could be prevented by sodium molybdate (which prevents receptor activation), suggesting that DNA binding was not dependent on the presence of GCs. Nuclear binding of a hormone-free GC receptor has also been observed following

depletion of cellular ATP with dinitrophenol (DNP; Mendel et al., 1986a), possibly due to a DNP-induced cellular stress response (Sanchez, 1992). In addition, cellular stress induced by heat shock (43°C) of a cell line (WCL2) that overexpresses the GC receptor, shows a time-dependent decrease in GC binding capacity in the soluble fraction of these cells, accompanied by a decrease in the amount of GC receptor protein (as measured with Western immunoblotting; Sanchez, 1992). This decrease in soluble GC receptors is also accompanied by an increase in nuclear localization of unliganded GC receptors, again confirming that heat shock is sufficient to cause a hormone-independent shift of this receptor protein from the soluble to the nuclear-bound fractions. In addition, the nuclear GC receptor in ATP-depleted cells is unable to bind steroid (Mendel et al., 1986a). It has been proposed that this nuclear receptor may represent a stage of the cycle following binding of the hormone-receptor complex, and prior to the regeneration of a hormone binding (i.e 10S) form of the receptor (Mendel et al., 1986a). These studies demonstrate the ability of the steroid-free GC receptor to bind to DNA following activation induced by cellular stress.

Therefore, the GC receptor can induce the transcription of GC target genes by binding to DNA sequences. However, GC receptors have also been shown to repress the transcription of other GC target genes by acting at "negative HREs". Such GC HREs are involved in the regulation of transcription of the pro-opiomelanocortin gene, the α-fetoprotein gene, and the prolactin gene (see Landers and Spelsberg, 1992 for review). Negative HREs are thought to bind GC receptors, thereby blocking the binding of an essential transcription factor to a DNA element adjacent to the HRE. An additional mechanism thought to be used by the GC receptor for gene repression is an interaction of the receptor protein directly with transcription factors (protein-protein interaction). Protein-protein interactions have been suggested to occur between the GC receptor and the transcription factor AP-1, a protein dimer composed of products of the *jun* and *fos* gene families (Diamond et al., 1990; Yang-Yen et al., 1990; Schüle et al., 1990). The

GC receptor appears to form specific complexes with c-*jun*, as *jun* homodimers, and with *jun-fos* heterodimers (Diamond et al., 1990). Other transcription factors such as the CACCC-box binding factor for the tryptophan oxygenase gene (Schüle et al., 1988) and the lymphocyte-specific Octamer factor 2A (Wieland et al., 1991) have been shown to exhibit direct protein-protein interactions with the GC receptor. In addition, regulation of gene expression by GCs may use "composite GC HREs", using both protein-protein interactions as well as binding of the GC receptor to its HRE (Diamond et al., 1990). Such regulation could account for either activation or repression of GC target genes, depending on the cell type and presence of specific transcription factors. Therefore, these studies demonstrate the complexity of the GC regulation of GC target genes, which involves both DNA and transcription factors.

### I.7.3 Cellular localization of GC receptors

Binding of the GC receptor to DNA in order to activate target genes requires prior hormone binding and most likely a translocation to the nucleus. This suggests that the receptor can be found in the cytoplasm of cells, where it will interact with hormone and become activated. Using immunocytochemistry, both estrogen and progestin receptors had been localized strictly to the nucleus in the absence of hormone (King and Green, 1984; Perrot-Applanat et al., 1985). For the GC receptor, however, the cellular location seems to be both cytoplasmic and nuclear (Antakly and Eisen, 1984; Picard and Yamamoto, 1987; Wikstrom et al., 1987; Qi et al., 1989). This finding was demonstrated in liver and pituitary tissue sections from intact rats, stained for the GC receptor using a polyclonal antibody and immunoperoxidase technique (Antakly and Eisen, 1984). Further support for a cytoplasmic localization of the GC receptor was provided in vitro by Picard and Yamamoto (1987) using a monoclonal antibody. Monkey kidney cell lines which lack detectable GC receptor were transfected with this receptor and maintained in culture. In the absence of serum in the culture medium, the unliganded

receptor was found predominantly in the cytoplasm, whereas the addition of 5% serum or DEX resulted in nuclear staining. Similar findings were obtained using a monospecific monoclonal antibody (mab 7; Wikstrom et al., 1987). The authors used a number of different permeabilization and fixation procedures, as well as examined the nuclear accessibility of the antibody, in order to more carefully examine the compartmental distribution of the GC receptor. In addition, the specificity of their antibody was confirmed, since preincubation with purified GC receptor prevented binding of the monoclonal antibody to the GC receptor in all cellular compartments. These studies demonstrated staining for the GC receptor in both cytoplasm and nucleus of rat hepatoma cells and normal hepatocytes cultured in medium with fetal bovine serum. The nuclear staining may have been due to the small amounts of GCs normally found in serum, since cells cultured in medium containing serum did not show enhanced nuclear staining when supplemented with DEX. On the other hand, cells cultured in medium treated with dextran-coated charcoal (to remove GCs) showed elevated nuclear staining following DEX treatment. This occurred at 37°C and not 4°C, suggesting a GC-mediated translocation of the receptor into the nuclear compartment.

Translocation as well as subsequent recycling of the GC receptor was demonstrated by Qi et al. (1989) using an oncogene-transformed rat cell line showing predominantly cytoplasmic staining of the hormone-free GC receptor (immunocytochemically labelled with the BuGR2 monoclonal antibody). In the presence of hormone, receptors were translocated to the nucleus, retained for approximately 12h and then returned to the cytoplasm. This series of events was found to occur in the presence of the protein synthesis inhibitor cycloheximide, suggesting that replenishment of GC receptors following nuclear binding occurs in the absence of de novo protein synthesis. This study supports the notion that following DNA binding GC receptors are recycled and reutilized. However, since immunocytochemistry was used to determine the subcellular distribution of the GC receptor, receptor reutilization was not demonstrated.

Nevertheless, these studies further support the existence of the GC receptor in the cytoplasmic compartment of cells.

Although it seems clear that the GC receptor can be found in the cytoplasm of cells, it has previously been argued that the cytosolic distribution of steroid receptors may be an artefact due to solubilization from the nucleus during the preparation of a cell cytosol (Gorski et al., 1984), or during fixation and incubation procedures required for immumocytochemical experiments (Wikstrom et al., 1987). However, this is unlikely since similar problems should arise with localization procedures for estrogen and progestin receptors, under similar conditions. Wikstrom and colleagues (1987) clearly show in their study, that using similar immunocytochemical procedures (i.e. fixation of both estrogen receptor- and GC receptor-containing cells with 2% paraformaldehyde and permeabilization with Triton X-100, prior to staining with antibodies), a strictly nuclear localization of the estrogen receptor is observed whereas the GC receptor is found both in the cytoplasm and nucleus. It remains possible that the presence of hormone-free receptors in the cytoplasm is due to production of new receptor proteins. Whether the GC receptor is found exclusively in one or the other cellular compartement may be dependent on the cell type being studied. For example, in the Chinese hamster ovary cell line WCL2, immumofluorescence studies have shown that the GC receptor is present solely within the nucleus in the absence of hormone (Sanchez et al., 1990). However, there does appear to be a consensus in the literature that this receptor, in most cell types, is localized within the cytoplasmic compartment in a resting state.

### I.7.4 Mechanism for the translocation of the GC receptor

Since the GC receptor appears to be localized to the cytoplasm of most cells, and the receptor must reach the DNA to exert its action once it is exposed to ligand, there must be a mechanism which regulates the movement of the receptor from the cytoplasm to the nucleus. There are two different possibilities that have been suggested to date. The first

is the existence of a nuclear localization signal for the ligand-bound GC receptor (Picard and Yamamoto, 1987; Wolff et al., 1987). This nuclear localization signal applies to all steroid receptors, since regardless of whether ligand-free receptors are localized to the nucleus or cytoplasm, all receptors are initially synthesized in the cytoplasm and must gain access to the nucleus (Wolff et al., 1987). Due to the homology with the SV40 T-antigen nuclear localization signal, the GC receptor nuclear transport signal has been identified as residues 491-498 (Wolff et al., 1987) or 493-499 (LaCasse and Lefebvre, 1991). Deletion mutation experiments have also identified similar sequences of the rat GC receptor cDNA as possible nuclear localization signals (NL1=497-524, NL2=550-795; Picard and Yamamoto, 1987). A chimera of the GC receptor hormone binding domain (comprising the putative nuclear localization signal NL2) was formed with ß-galactosidase, a normally cytoplasmic protein. This chimera was able to translocate to the nucleus in response to GC exposure (Picard and Yamamoto, 1987), suggesting that there is a nuclear localization signal found in this portion of the GC receptor.

Nuclear localization sequences have also been identified in the receptors belonging to the steroid receptor superfamily (see LaCasse and Lefebvre, 1991). These nuclear localization signals must recognize specific sequences on the nuclear envelope in order to enter the nucleus. Recognition sequences for the GC receptor have been identified on the nuclear envelope as peptides of 60 and 76 kDa (LaCasse and Lefebvre, 1991). According to those authors, ligand binding presumably unmasks the nuclear localization signal allowing it to bind to the 60 kDa general nuclear docking protein on the nuclear envelope. The receptor is then thought to bind to the 76 kDa nuclear importer peptide, which may be specific for the transported molecule, in order to gain access to the nucleus.

For the GC receptor, in contrast to receptors for estrogens and progestins, the hormone binding domain of the receptor seems to mask the nuclear localization signal (Ylikomi et al., 1992) and may thus account for the cytoplasmic location of this receptor. This conclusion was drawn from the following two sets of studies: 1) Chimeric receptors

of the progestin receptor, with a substitution of the nuclear localization signal for that of the GC receptor, resulted in nuclear localization indistinguishable from the wild type progestin receptor. This suggested that the signals for both receptors are of equal strengths in their ability to promote nuclear entry. 2) A chimera of the estrogen receptor sequence 1-281 and the hormone binding domain of the GC receptor, in the absence of GC, resulted in less nuclear labelling of the construct (10% nuclear labelling) compared to an estrogen receptor containing only sequence 1-281 (62% nuclear labelling). However, in the presence of GC, this chimera exhibited equivalent nuclear labelling compared to the wild-type estrogen receptor. Both of these sets of experiments suggest that the nuclear localization signal for the GC receptor is responsible for entry into the nucleus and is only able to act once unmasked by the presence of hormone. However, as for the early theory of unmasking the DNA binding domain following hormone binding, the nuclear localization signal may be masked by the heat shock proteins which are only dissociated following hormone binding.

The second possibility regulating the entry of the GC receptor into the nucleus is by transportation of the receptor by cytoskeletal proteins. One possible candidate is cytoplasmic microtubules, since the GC receptor has been found to be codistributed with these proteins in human fibroblasts (Akner et al., 1990; Akner et al., 1991). Translocation of the GC receptor into the nucleus following GC exposure of COS7 or CV-1 cells has been reported to occur within minutes (Picard and Yamamoto, 1987). This efficient translocation may be occuring via a transport mechanism involving cytoskeletal proteins (Akner et al., 1991). In addition, Akner and colleagues (1991), using double labelling, have shown that the distribution of GC receptor follows the redistribution of microtubules during the mitotic process of the cell cycle, suggesting that the receptor may use these microtubules for its movement into the nucleus following hormone binding. However, no direct evidence for microtubule-assisted entry into the

nucleus is as yet available to solely account for the entry of the GC receptor into the nucleus.

### I.7.5 Membrane effects of GCs

The classical view of steroid hormone action is that steroids alter the expression of target genes, an event which occurs over several hours. However, non-genomic effects of steroids have been observed, occuring within a matter of minutes. For example, using cultured guinea pig chromaffin cells, Inoue and Kuriyama (1989) have shown a decrease in the ACh inward current following application of 25 μM DEX. This decrease occurs within seconds, and is probably not due to nonspecific membrane effects of the steroid since DEX had no effect on the γ-amino butyric acid (GABA) inward current. In addition, GCs (nmole doses given i.p.) have been shown to suppress male amphibian sexual behaviour within 8 minutes of injection (Orchinik et al., 1991). In mouse and rat brain synaptosomes, GCs have been reported to stimulate the uptake of radiolabelled tryptophan (Neckers and Sze, 1975; Towle and Sze, 1983), with half-maximal saturation of the GC membrane receptor ($Kd=10^{-7}$ M) correlating with a 50% increase in the uptake of the amino acid (Towle and Sze, 1983). These studies suggest that GCs can have a physiological role by binding to membrane receptors, since the effects of GCs are rapid and are therefore unlikely due to an action on cytosolic GC receptor-mediated gene regulation.

Plasma membrane GC receptor binding has been measured in the CNS (Towle and Sze, 1983; Orchinik et al., 1991), and liver (Quelle et al., 1988). Receptors in brain synaptosomes can lose their binding capacity if the membrane fraction is first pretreated with phospholipase $A_2$ or C, suggesting that these binding sites may involve a lipoprotein (Towle and Sze, 1983). However, these binding sites are stable at 30°C, unlike cytosolic GC receptors, and have a differential distribution compared to cytosolic receptors in specific brain regions (Towle and Sze, 1983). In addition, these membrane GC receptors

can bind to DEX, ruling out the possibility that these sites are CBG (Towle and Sze, 1983). Therefore, these membrane receptors appear to be a distinct population of GC receptors. The receptor $K_d$s for membrane GC receptors have been reported as 0.5 nM for amphibians ([$^3$H]-B; Orchinik et al., 1991), and ≈100 nM ([$^3$H]-B and [$^3$H]-DEX; Towle and Sze, 1983), 180 nM ([$^3$H]-cortisol) and 420 nM ([$^3$H]-DEX; Quelle et al., 1988) for rodents. These $K_d$s are higher than those found for intracellular GC receptors (low nanomolar range), suggesting that membrane receptors may be functional under conditions of stress where GC levels reach the high nanomolar range (Quelle et al., 1988).

For the adrenal chromaffin cells in culture, the Ki for DEX for inhibition of the inward ACh current was ≈10 μM (Inoue and Kuriyama, 1989), a concentration consistent with the physiological range of concentrations of GCs seen by the adrenal medulla due to its close proximity to the adrenal cortex ($10^{-6}$ to $10^{-4}$ M; Kitay, 1961; Peytremann et al., 1973; Guidotti and Costa, 1974; Jones et al., 1977; Engeland et al., 1989). The inhibition of the inward ACh current by DEX application occured even following prior intracellular perfusion of the cell with steroid (Inoue and Kuriyama, 1989). This suggested that intracellular GC receptors were not responsible for the inhibition of the ACh inward current, and that this inhibition was mediated by chromaffin cell membrane GC receptors. However, it is unclear at this time what the functional role of the putative adrenomedullary membrane GC receptor may be.

In the present thesis however, we are measuring an effect of GCs that requires a time delay, suggesting that the receptor we are dealing with is a classical steroid receptor, requiring gene-mediated events (see above) in order to observe function. The cytosolic adrenomedullary receptor has been suggested to be of the type II subtype of GC receptors (Kelner and Pollard, 1985). Therefore, before the literature on the medullary receptor is discussed, a brief overview of the classification of GC receptor subtypes will be helpful.

### I.7.6 GC receptor subtypes

In the mid-1970's, DeKloet and colleagues reported that following systemic injection of rats with radiolabelled B or DEX, different brain areas had distinct preferential uptake of one steroid (DeKloet et al., 1975). This led to the suggestion that there were two separate classes of receptors for GCs. The specificity of these subtypes to different hormones was demonstrated using biochemical and autoradiographic techniques (Ermish and Ruhle, 1978; Birminghan et al., 1979; Beaumont and Fanestil, 1983; Coirini et al., 1985; Reul and DeKloet, 1985). This resulted in a classification of the receptors for B as the type I and type II subtypes. In addition, the advent of the Roussel-Uclaf compounds, which are highly specific ligands for the receptor subtypes, provided further tools for this characterization. These studies demonstrated that the type I receptor is the kidney mineralocorticoid receptor, with high affinities for aldosterone ($K_d \approx$ 2 nM) and B ($K_d \approx$0.5-1 nM). Competition for [$^3$H]-aldosterone binding to rat hippocampal receptors showed that the type I (mineralocorticoid) receptor-specific ligand RU 26752 efficiently inhibited binding of the radioligand whereas the type II-specific ligand RU 28362 was unable to compete for radioligand binding (Coirini et al., 1985). B binds to the type II (GC) receptor with a lower affinity ($K_d \approx$2.5-5 nM) compared to its affinity for the type I receptor (Reul and DeKloet, 1985). The type II receptor binds with higher affinity to DEX than does the type I receptor, and RU 28362 ($K_d \approx$0.5-2 nM) binds specifically to the type II receptor. Therefore, there are two subtypes of receptors which are able to bind to GCs in the brain. These subtypes are also used to pharmacologically distinguish between receptors for GCs and mineralocorticoids in non-CNS tissues such as kidney, liver and adrenal. In the adrenal medulla, the characterization of the subtype of GC receptor is of interest given that the medulla is exposed to both GCs and mineralocorticoids secreted from the adrenal cortex.

### I.7.7 The cytosolic adrenomedullary GC receptor

The regulation of adrenomedullary enzymes by GCs has been known for over 25 years. However, it was not until 1985 that two groups independently published results on the adrenomedullary cytosolic GC receptor. Specific, soluble binding of [$^3$H]-DEX was observed in cytosolic fractions of cultured bovine adrenal medullary cells (Nawata et al., 1985; Kelner and Pollard, 1985). In the case of Nawata and colleagues, the Kd observed was 35 nM. This is approximately 10-fold higher than the $K_d$ for the GC receptor reported in a number of other tissues. However, in the binding assay for GC receptors, the presence of molybdate is essential in order to stabilize the receptor and prevent activation (Kalimi and Hubbard, 1983; Mitchell et al., 1986), an element omitted from the binding study of Nawata et al. (1985). Kelner and Pollard (1985), whose binding assay included sodium molybdate and dithiothreitol [both of which stabilize the receptor protein, one by preventing activation of the receptor (molybdate), the other by preventing breakdown of the sulfite bridges maintaining the 3D conformation of the receptor], observed a $K_d$ of 1.28 nM, similar to the reported $K_d$s for the GC receptor of other tissues. In addition, competition of [$^3$H]-DEX binding by various steroids suggested a receptor of the type II subtype. However, these studies were performed prior to the availability of the highly specific type II or GC receptor ligand RU 28362 (Philibert and Moguilewsky, 1983; Coirini et al., 1985), leaving open the possibility that the measured receptor was a steroid receptor with affinity for DEX, such as the type I mineralocorticoid receptor (Luttge et al., 1989). The present thesis sought to clarify this point and fully characterize the dynamics of this receptor, given the adrenomedullary environmental exposure to GCs several orders of magnitude higher than the general circulation. In addition, the regulation of this receptor by a variety of influences was of interest given the demonstration in a number of other tissues of regulation by GCs and by cyclic nucleotides, both of which are elevated in the adrenal medulla during a stress response.

### I.7.8 Regulation of the GC receptor

The magnitude of the biological response to GCs has been shown to be proportional to the levels of GC receptors expressed within the cell (Bourgeois and Newby, 1979; Vanderbilt et al., 1987; Distelhorst, 1989; Dong et al., 1990; Tanaka et al., 1992). For example, in human mononuclear and polymorphonuclear leukocytes, the levels of GC receptors correlated ($r=0.95$) with the inhibition of glucose uptake into these cells (Tanaka et al., 1992). This inhibition was more marked following an 8-bromo-cAMP-induced increase in the levels of GC receptor binding, suggesting a direct relationship of GC receptor levels to cellular function. Therefore, the sensitivity of a given cell to GCs will be affected by changes in the concentrations of GC receptors. In adrenomedullary cells, changes in receptor capacity could alter the output of CAs, given that GCs are necessary for the maintenance of levels of CA biosynthetic enzymes. Therefore, in the present thesis, we have examined the regulation of the adrenomedullary GC receptor.

#### I.7.8.1 Autoregulation

The GC receptor has been shown to be homologously downregulated in the CNS in vivo (Sapolsky et al., 1984; Meaney and Aitken, 1985) and in vitro in a number of cells lines such as GH1 (McIntyre and Samuels, 1985), HeLa (Cidlowski and Cidlowski, 1981) and AtT-20 (Svec and Rudis, 1981; Svec, 1985a; 1985b). The ability of GCs to downregulate their own receptor levels does not result in total depletion of receptors. Both in vivo and in vitro, the extent of receptor depletion reaches a floor value of between 20 and 40% of total receptor content (Svec and Rudis, 1982; Svec, 1985b; Sapolsky et al., 1984). Receptor depletion in response to various GCs was found to be of the same order of binding preference for the receptor (Svec, 1985b). In addition, depletion

occurred with concentrations of steroids of the same order as the affinity for the receptor, suggesting that depletion is a receptor-mediated event (Svec, 1985b).

However, these studies used soluble receptor binding assays in order to measure GC receptor levels. Therefore, any GC receptor that is in a transformed state and not ligand bound (such as might occur during receptor recycling), cannot be measured with radioligand binding techniques. More direct evidence for a GC receptor-mediated downregulation of the GC receptor was reported in studies using antibodies to the receptor. Okret et al. (1991) reported a time-dependent decrease in hepatic levels of both GR protein and mRNA following in vivo treatment with DEX. Maximum decreases in GC receptor protein and mRNA were observed at 18-24h following DEX treatment, returning to original levels 48h hours later. Earlier in vitro studies by the same group had shown a similar GC-induced decrease in GC receptor mRNA (Okret et al., 1986). Strong evidence for a direct regulation of GC receptor levels by GCs was provided by Okret et al. (1986) who demonstrated that purified rat liver GC receptors bound to a GC receptor cDNA clone, indicating that GCs can cause a downregulation of their own receptor.

The mechanisms of GC-induced autoregulation were suggested by the following results. In vivo, DEX decreased the rate of transcription of the GC receptor gene as measured by nuclear run-on assays (Okret et al., 1986). In vitro, in the absence of GCs, the half-life for the receptor was found to be ≈19-25h, whereas in the presence of GCs the half-life was ≈9.5-11h (McIntyre and Samuels, 1985; Okret et al., 1991). In addition, Govindan et al. (1991) showed that a region upstream of the transcription start site for the human GC receptor mRNA was required in order to observe homologous downregulation. Therefore, the mechanism of autoregulation of receptor protein levels appears to be due to both transcriptional repression of GC receptor gene expression as well as to post-transcriptional events involving translation and turnover of the GC receptor protein.

### I.7.8.2 Regulation by cyclic nucleotides

Regulation of GC receptor and mRNA levels by cyclic nucleotides has been studied in a number of cell types such as human skin fibroblasts (Oikarinen et al., 1984) and mononuclear leukocytes (Tanaka et al., 1992), murine lymphoma cells (Gruol et al., 1986; Gruol et al., 1989), rat HTC cells (Dong et al., 1989; Okret et al., 1991), rat hippocampal cells (Mitchell et al., 1992), and AtT20 cells (Sheppard et al., 1991) in culture. In human skin fibroblasts, using ligand binding assays, Oikarinen et al. (1984) showed that dibutyryl-cAMP treatment increased while -cGMP decreased the number of GC receptor molecules per cell, in a concentration-dependent manner. In addition, during cell growth, the levels of GC receptor correlated (r=.87) with the cAMP/cGMP cellular ratio (Oikarinen et al., 1984). This was one of the first studies to suggest that the GC receptor could be regulated by intracellular levels of cyclic nucleotides and that the ratio of these nucleotides may be a regulatory parameter. However, no effect of cGMP on GC receptor binding levels were observed in murine lymphoma cells (Gruol et al., 1986) and the effect of this cyclic nucleotide was not studied in most of the papers which have examined the regulation of GC receptors by cAMP.

Measuring binding of radiolabelled GCs is an estimate of the receptor capacity of the cell. Using this method, the above studies (with the exception of Sheppard et al., 1991) showed cAMP-induced increases in GC receptor levels. A more precise method of studying upregulation of the number of receptors is by using antibodies to the receptor protein. This technique allows measurement of all three possible states of the receptor (steroid-free receptors, activated hormone-bound receptors and transformed but not hormone-bound receptors). Dong et al. (1989) have used this method to measure total levels of GC receptor protein in rat liver HTC cells. Forskolin treatment, which increases intracellular levels of cAMP, was able to increase total GC receptor content. In addition, treating HTC cells with 8-bromo cAMP increased the mRNA levels for the GC receptor as well as increased the half-life of the receptor mRNA (Dong et al., 1989). Therefore,

the action of both GCs and cAMP on the regulation of the GC receptor appear to be partly via a post-transcriptional mechanism.

The importance of cyclic nucleotide-dependent protein kinases in the regulation of GC receptors has been studied by a number of groups. Gruol et al. (1986) reported that murine lymphoma cell variants with a reduced level of cAMP-dependent kinase activity were unable to respond to a cAMP-induced regulation of the GC receptor. The basal levels and nuclear translocation of GC receptors in cells without the kinase were not different from wild type cells. In hippocampal cells in culture, we have previously shown that 8-bromo cAMP increases the levels of GC receptors to the same extent as does serotonin (Mitchell et al., 1992). In addition, half-maximal induction of GC receptor levels by serotonin was reduced significantly by the concurrent addition of H-8, an inhibitor of cyclic nucleotide-dependent protein kinases (Mitchell et al., 1992). Therefore, these studies suggest that cAMP-induced regulation of the GC receptor in a number of cell types seems to require a functional kinase activity.

In all cell types studied to date, elevation of cAMP has been shown to increase the levels of GC receptor protein and/or mRNA. However, one exception is the mouse anterior pituitary tumour AtT-20 cell line, where both forskolin and/or 8-bromo cAMP decrease the levels of nuclear binding of [$^3$H]-DEX and GC receptor mRNA (Sheppard et al., 1991). CRF, which acts by increasing levels of cAMP, was able to decrease GC receptor mRNA, as well as the levels of [$^3$H]-DEX in whole cell, nuclear and cytoplasmic fractions. This study suggested that there may be cell-specific factors which are involved in the cAMP-mediated regulation of the GC receptor. In the present thesis, our results concur with those found in the AtT-20 cell line, since in adrenal medullary cells 8-bromo cAMP causes a reduction in the binding capacity of GC receptors.

In summary, this chapter has dealt in general with stress and the adrenal gland, with special attention given to the relationship between adrenocortical GCs and adrenomedullary CAs, and to the pharmacology of the GC receptor involved in this

interaction. Given this background state of knowledge, the following section will briefly outline the research undertaken in the present thesis.

## Aims and Objectives

The following constitues connecting text providing logical bridges between the different papers which are presented as Chapters II through V, as per the Faculty of Graduate Studies and Research "Guidelines Concerning Thesis Preparation".

The literature reviewed above helps us to understand the importance of examining in greater detail the characterization, regulation and function associated with GC receptors in the adrenal medulla. GC receptors had been measured in adrenal medullary cells. However, it was not clear as to the subtype of corticosteroid receptor present in such cells. Chapter II clarifies this point by using a specific ligand for the GC receptor and by providing detailed competition studies using a number of different steroids. In addition, the dynamics of the medullary receptor had not been studied. Therefore, it was not known whether this receptor behaved like the GC receptor found in the nervous system and liver, i.e. whether the adrenomedullary GC receptor would translocate at low nM levels of steroid given its exposure to 100-fold or higher levels of hormone. In addition, it was not known whether translocation of the adrenomedullary GC receptor would occur at higher concentrations of GCs, given the high concentrations seen by the medulla basally. These points have been addressed in Chapter II, which demonstrates that the adrenomedullary GC receptor dynamics at low concentrations of GCs are similar to the classical type II GC receptor of the CNS. We have also shown that the adrenomedullary GC receptor dynamics are novel in that a further translocation is observed at higher concentrations of steroid. In addition, Chapter II confirms the effect of long-term GC exposure on the regulation of PNMT activity and provides novel evidence for a functional effect of short-term GC exposure on the activity of PNMT.

In Chapter III, the regulation of levels of adrenomedullary GC receptors has been studied. The adrenal medulla is exposed to high levels of GCs due to its proximity to the cortex, and to neurotransmitters/neuromodulators due to innervation by the splanchnic nerve. Therefore, it was of interest to know whether regulation of the receptors by GCs or by second messengers used by many of the neurotransmitters released from the splanchnic nerve, could regulate levels of GC receptors in the adrenal medulla. In other tissues, this type of receptor regulation had been observed. Chapter III provides evidence for a regulation of GC receptor binding by analogues of the cyclic nucleotides, cAMP and cGMP, as well as by GCs themselves. Since GCs are known to play an important role in the regulation of PNMT activity and thus A synthesis, regulation of the receptor involved in this functional response of the medulla could affect the animal's response to stressful stimuli. This has been addressed in Chapter III, which shows that decreasing levels of GC receptor binding by prior treatment with a cAMP analogue results in a compromised ability of the cell to respond to a GC pulse by increasing PNMT activity.

Finally, in Chapters IV and V, the functional implication of the interaction between the adrenal cortex and medulla was studied in great detail with a focus on the time course of the regulation of PNMT by GCs. GCs had been known to regulate this enzyme following long-term exposure to elevated GC levels both in vivo and in vitro. However, short-term elevations in GCs, such as occur during acute stressors in vivo, were not thought to regulate PNMT activity. In Chapter IV, using an in vitro culture model of adrenal medullary cells, we have examined the ability of short-term exposure of GCs to regulate this enzyme following a lag period. We provide the literature with novel evidence for such a regulation, showing that pulses as short as 15-30 min can increase the activity of PNMT with a steroid-free lag period of at least 18h. In addition, we extend these findings to include a regulation following short-term exposure of cells to the cholinergic agonist nicotine, and explore the time delays following a combination treatment with GCs. These results point to a more dynamic view of the regulation of CA

biosynthesis by mimicking acute stress-induced changes in both the hormonal and neural signals. In Chapter V, we have also studied the temporal pattern of both PNMT and TH activities in vivo following a short-term mild stressor, and have examined the importance of the hormonal signal in the regulation of these enzymes. We study the effect of a single episode of acute stress (20 min restraint) on enzyme activities in intact rats, as well as in rats with suppressed endogenous corticosterone release, over a time course of hours to days. Therefore, the studies presented in Chapters II through V have furthered the field of GC regulation of CA biosynthesis, and have shown that this regulation is more dynamic than was classically thought.

# Chapter II. Adrenal Phenylethanolamine N-Methyltransferase Induction in Relation to Glucocorticoid Receptor Dynamics: Evidence that Acute Exposure to High Cortisol Levels is Sufficient to Induce the Enzyme

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Abbreviations and trivial names used: PNMT, phenylethanolamine N-methyl transferase; GC, glucocorticoid; DEX, dexamethasone; DMEM, Dulbecco's Modified Eagle's Medium; $K_d$, dissociation constant; $B_{max}$, maximal binding capacity; RU 38486, 17ß-hydroxy-11ß,-4-dimethylaminophenyl-17a -propynyl estra-4,9-diene-3-one; RU 28362, 11ß, 17ß-dihydroxy-6-methyl-17a-(propionyl)-androsta-1,4,6-triene-3-one.

## Abstract

Glucocorticoids (GCs) are thought to regulate, in a permissive fashion, the basal activity of adrenal medullary phenylethanolamine N-methyltransferase (PNMT). However, it is unclear whether a large short-term increase in GC release, such as occurs during an acute stress response, may also play a role in PNMT regulation. The present study investigated how the GC influence over PNMT activity varies in relation to dynamic changes in the hormone-receptor signal. Using [$^3$H]-dexamethasone (DEX) and [$^3$H]-RU 28362 as radioligands, we have confirmed the presence of GC receptors in bovine adrenal medullary cells. A decline in soluble GC receptor sites and an increase in nuclear uptake of [$^3$H]-DEX was found in response to GC levels as low as $5x10^{-8}$ and was concentration dependent. The loss of soluble sites plateaued between $5x10^{-8}$ and $10^{-6}$ M cortisol with a further loss occuring at $10^{-5}$ M and again at $10^{-4}$ M. The functional consequence of GC receptor binding was confirmed by measuring PNMT activity following 3 day exposure to cortisol. The pattern of PNMT induction was similar to that seen with GC receptor occupancy; at cortisol concentrations between $10^{-8}$ and $10^{-5}$ M, PNMT induction was at a plateau, with a further increase in activity at $10^{-4}$ M. The increase in PNMT activity following 3 day exposure to low ($10^{-7}$ M) and high ($5x10^{-5}$, $10^{-5}$ M) cortisol was blocked by the GC receptor antagonist RU 38486, suggesting a GC receptor mediated event. Finally, a short (2h) pulse of GCs, that mimics the time course of physiological elevation of GCs following acute stress, elevated adrenal medullary PNMT activity measured 3 days later. Therefore, our results provide novel evidence that short-term exposure of adrenal medullary cells to high cortisol levels can elevate PNMT activity.


**Key Words:** glucocorticoid receptors, phenylethanolamine N-methyltransferase, adrenal medulla.

**Running title:** Glucocorticoid receptors and adrenal PNMT

## Introduction

Glucocorticoids (GCs) are known to regulate the synthesis and release of neurohormones and neurotransmitters (see McEwen et al., 1986, for a recent review). As early as 1965, Wurtman and Axelrod (1965) showed that hypophysectomy reduced levels of adrenal medullary phenylethanolamine N-methyltransferase (PNMT, E.C. 2.1.1.28), the enzyme catalyzing the conversion of noradrenaline to adrenaline. The effects of hypophysectomy were reversed by administration of the synthetic GC, dexamethasone (DEX), suggesting that GCs regulate levels of adrenal PNMT. Later experiments with cultures of isolated bovine adrenal medullary cells showed that long-term (18-48h) exposure of medullary cells to GCs resulted in an increase in PNMT activity (Hersey and DiStefano, 1979; Kelner and Pollard, 1985). This finding is consistent with the suggestion that GCs regulate adrenal PNMT by interacting directly with GC receptors within the chromaffin cells.

In 1985, Kelner and Pollard (1985) and Nawata and colleagues (1985) demonstrated the presence of specific, soluble [$^3$H]-DEX binding sites, with $K_d$s of 1.28 nM and 35 nM respectively, in cultures of bovine adrenal medullary cells. The profile of displacement of binding by various steroids was consistent with binding to type II (or GC) corticosteroid binding sites. [However, these studies were carried out before specific GC receptor ligands such as RU 28362 (Philibert and Moguilewsky, 1983; Coirini et al., 1985) became available to allow complete characterization of the mineralocorticoid (type I) and GC corticosteroid receptor subtypes, nor did these studies investigate which medullary cell types contain GC receptors].

Despite the accumulating evidence that GCs play an important role in regulating adrenal PNMT, the prevailing notion has been that GCs may regulate basal levels of PNMT in a permissive fashion with the higher levels of GCs released during an acute stress response having little additional effect on PNMT (Pohorecky and Wurtman, 1971; Hersey and diStefano, 1979). This idea was suggested by two sets of observations.

Firstly, due to the anatomical association between the adrenal medulla and the adrenal cortex, the medulla is exposed to very high levels (6 to 10 x $10^{-5}$ M in the rat; Jones et al., 1977) of GCs. These levels are much higher than the nM GC levels needed to occupy and activate GC receptors in other tissues. Secondly, the time of exposure to GCs (at least 18h) necessary to observe effects on PNMT in both in vivo and in vitro experiments (Pohorecky and Wurtman, 1971; Hersey and diStefano, 1979; Kelner and Pollard, 1985) is much greater than the duration of GC elevation measured during an acute stress response.

The aim of the present study was to investigate how the GC influence over PNMT activity varies in relation to dynamic changes in the hormone-receptor signal. Using the range of GC concentrations seen by the adrenal medulla basally and during stress conditions, we have examined changes in PNMT activity in relation to various levels of GC receptor activation (characterized in terms of receptor occupancy and translocation). We have also observed effects of a short pulse of cortisol, that mimics the time course of physiological elevation of GCs following a single episode of acute stress, on the GC receptor signal and on adrenal medullary PNMT activity measured 3 days later. Our results support the idea that GC receptors may play a role in regulating not only basal levels of catecholamines but also in further modulating catecholamine synthesis during short-term exposure to high levels of GCs, such as occurs in the acute stress response.

## Experimental Procedures

### Cell Culture

Adrenal medullary cells were isolated by retrograde perfusion of bovine adrenal glands with collagenase and DNase I, and purification of isolated cells on Percoll density gradients as described (Trifaró et al., 1978; Trifaró and Lee, 1980). Cells were plated on collagen-coated 35 $mm^2$ culture plates ($10^6$ cells/plate) or on 24 well plates (2.5 x $10^5$

cells/well) and maintained at 37°C in 10% $CO_2$ in air in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% fetal calf serum, 1% glucose, 100 ug/ml penicillin, 100 ug/ml streptomycin, 5 ug/ml gentamycin sulfate, 10 Units/ml mycostatin and $10^{-5}$ M cytarabine (Cytosar, Upjohn; used to prevent growth of rapidly dividing non-chromaffin cells). For all studies, the cells were used between day 6-10 of culture and were placed in serum-free, steroid-free medium [50% DMEM, 50% Ham's F12 Nutrient Mixture (GIBCO)] with antibiotics and Cytosar for at least 2 days before use. The data for each set of experiments were collected from at least 3 cell culture preparations with the exception of the differential plating study which was from 2 different cell culture preparations.

Medullary cell cultures prepared by the above protocol contain approximately 85-90% chromaffin cells and 10-15% non-chromaffin cells (cortical and endothelial cells, fibroblasts, Unsicker and Müller, 1981; Banerjee et al., 1985). In one set of studies, isolated medullary cells were further purified into highly chromaffin-enriched and non-chromaffin-enriched cell populations by differential plating (Waymire et al., 1983). Isolated medullary cells were placed into 80 $cm^2$ plastic flasks (20 x $10^6$ cells/25 ml of serum-free medium without Cytosar/flask). The flasks were incubated at 37°C for 3 hours, and the chromaffin cells were decanted and plated as above. The non-chromaffin cells were removed with 0.125% trypsin (type I, Sigma) at 37°C for 5 minutes and then plated as above, except that the medium contained no Cytosar.

## GC receptor binding experiments

Percoll-isolated adrenal medullary cells in culture were washed with an ice-cold isotonic salt solution (wash buffer, 154 mM NaCl, 5.6 mM KCl, 10mM glucose, 5 mM trizma acetate; pH=7.5) and scraped into ice-cold buffer [TEDGM=30 mM tris HCl, 1 mM disodium EDTA, 1 mM dithiothreitol, 10% v/v glycerol and 10mM sodium molybdate, pH=7.4], at a concentration of $10^6$ cells/100 ul TEDGM and sonicated on

ice. The homogenate was centrifuged at 0-2°C for 45 minutes at 105 000*g* . GC receptor binding was carried out using methods established by Kalimi and Hubbard (1983). For saturation experiments, an aliquot (150 ul) of the supernatant (cytosol extract) was incubated with 100 ul aliquots of TEDGM containing either [1,2,4 - $^{3}$H]-dexamethasone (DEX; specific activity = 46 Ci/mmole; Amersham, Oakville, Ontario) or [6-methyl-$^{3}$H]-RU 28362 (specific activity = 77 Ci/mmole; New England Nuclear, Boston, Mass.) in a final concentration range of 0.1-15 nM at 0-4°C. Incubation was for 20-22 hours. A 20 h incubation allows endogenous steroid bound to GC receptors to exchange maximally with radiolabelled steroid, and binding is stable at this time point; thus this protocol measures the total population of non-transformed soluble GC receptors (Kalimi and Hubbard, 1983). Following the incubation, bound steroid was separated from free on Sephadex LH20 (Pharmacia Fine Chemicals, Dorval, Qué.) columns (7 x 1 cm, equilibrated with TEDGM) . Aliquots (100 ul) of the incubates were washed into the columns with 100 ul TEDGM and bound steroid was eluted with 500 ul TEGM (TEDGM without dithiothreitol) 30 minutes later. Radioactivity in the eluate was counted in 4.5 ml Liquiscint (National Diagnostics, Somerville, NJ) at 30% efficiency. For the competition study, a saturating (12 nM) concentration of [$^{3}$H]-DEX was used. In some experiments, single-point incubations with a saturating concentration of [$^{125}$I]-DEX (12 nM) or [$^{3}$H]-RU 28362 (7 nM) were used. Non-specific binding was determined using parallel incubations with a 500 fold excess of unlabelled cortisol (the major endogenous bovine GC). Binding data from saturation experiments were analyzed by Scatchard (1949) analysis using the McPherson Ligand computer program. Protein was measured by the method of Bradford (1976). The data are presented as femtomoles bound per milligram of soluble protein.

## Translocation of GC Receptor and Nuclear Uptake of [$^3$H]-DEX

To quantify translocation of the GC receptor from a soluble to an insoluble compartment in response to GCs, medullary cells were incubated for 45 min at 37°C with serum-free medium containing unlabelled cortisol. The cortisol-containing medium was removed, the cells were washed twice with serum-free medium and harvested at various time points following removal of cortisol. A soluble fraction was prepared from the cells, aliquots were incubated with a saturating concentration (12 nM) of [$^3$H]-DEX, and soluble GC receptor binding assessed, as described.

To measure nuclear uptake of GC receptor, medullary cells ($2x10^6$ cells/point) were incubated with [$^3$H]-DEX in a concentration range of $10^{-9}$ to $10^{-7}$ M, for 45 min at 37°C. (Higher concentrations were not tested since levels of steroid in ethanol vehicle required to assess non-specific binding compromised cell viability). To determine non-specific uptake, parallel plates were incubated with [$^3$H]-DEX and $10^{-4}$ M of unlabelled cortisol. Following incubation, the cells were washed twice and harvested into ice cold wash buffer ($2 \times 10^6$ cells/1 ml). The whole cells were centrifuged at 800*g* for 5 min, resuspended in 1.5 ml hexylene glycol buffer [(1M hexylene glycol, 0.1 mM $MgCl_2$, 2 mM dithiothreitol, 5 mM EGTA, 1 mM piperazine-N,N´-bis-[2-ethane-sulfonic acid] (Pipes); pH=7.5)] and a nuclear fraction prepared according to the method of Wray et al. (1977). Briefly, the resuspended cells were sonicated and centrifuged at 1500*g* for 10 minutes, the pellet resuspended in 1 ml hexylene glycol buffer and recentrifuged. The resulting pellet was resuspended in a 2 M sucrose buffer containing 50 mM Tris HCl, 2.5 mm KCl, 2 mM MgCl2, 1 mM dithiothreitol (pH=7.5), and centrifuged at 80 000*g* for 30 min. The supernatant was removed, and the tritium label was extracted from the pelleted nuclear fraction overnight with 1 ml ethanol. Radioactivity in 500 ul of the ethanol extract was counted in 4.5 ml of Liquiscint, and the volume of ethanol remaining was measured to assess degree of evaporation during the overnight extraction.

### Phenylethanolamine N-methyltransferase (PNMT) activity

Adrenal medullary cells plated in 24 well culture dishes and maintained in serum-free medium were treated with cortisol (and/or RU 38486) for the amount of time indicated in the figure legends. (RU 38486 was generously provided by D. Philibert, Roussel-Uclaf, Romainville, France). Following treatment, each well was scraped into 150 ul of water, subjected to a freeze-thaw cycle (-80°C) and centrifuged at 12 800*g* for 10 min. Aliquots (90 ul) of the supernatant were assayed for PNMT activity as described by Pollard et al. (1979). The assay is based on catalysis of the methylation of substrate (phenylethanolamine, 1.8 mM) by [6-methyl-$^3$H]-S adenosyl methionine (10 μM) and extraction of tritium labelled methyl product into toluene/isoamyl alcohol. Protein was measured by the method of Lowry et al. (1951). In control cultures containing ethanol in a concentration equal to the highest used to dissolve steroid, PNMT activity was unaffected. Adrenal medullary cells under control conditions typically contain approximately 35 pmol/30 min/μg protein of PNMT activity.

### Measurement of Catecholamines

Catecholamines were measured by HPLC with electrochemical detection. Adrenal medullary cells ($10^6$ cells/2.8 ml) were scraped into 0.1 M perchloric acid containing 0.1 mM disodium EDTA, using epinine as the internal standard, sonicated and centrifuged at 7000*g* for 5 minutes. An aliquot of the supernatant was assayed for catecholamines. The conditions used for HPLC separation and detection of adrenaline, noradrenaline, and dopamine were as described by Boksa (1990) except that the mobile phase contained 100 mg/ml sodium octyl sulfate.

### Statistical Analyses

Raw data were analysed using one way analysis of variance or Student's t-test, and comparisons performed with post hoc Newman-Keul's ($p<0.05$), where appropriate.

Dose response of translocation and PNMT data were expressed as a percent of control, and statistical differences were tested using non-parametric statistics (Mann-Whitney two sample test, $p<0.05$).

## Results

### Characterization of GC Receptor Binding

Initial experiments confirmed the presence of GC receptors in cultures of bovine adrenal medullary cells. Saturation experiments were done (Fig 1) with both [$^3$H]-DEX, an agonist with high affinity for the classical GC receptor, and [$^3$H]-RU 28362, a pure GC receptor agonist (Philibert and Moguilewsky, 1983; Coirini et al., 1985). Scatchard analyses of experiments done with either radioligand revealed a single population of saturable receptors with average dissociation constants ($K_d$) = 3.32 ± 0.57 nM and 1.37 ± 0.22 nM (means ± S.E.M.s from n=8-9 experiments) for [$^3$H]-DEX and [$^3$H]-RU28362, respectively. These $K_d$s differed significantly ($p<0.05$), a finding consistent with the reported higher affinity of [$^3$H]-RU28362 for the GC receptor. The average maximal binding capacities ($B_{max}$) for the two ligands [105.9 ± 13.3 and 88.0 ± 7.8 fmole/mg protein (means ± S.E.M. from n=8-9 experiments) for [$^3$H]-DEX and [$^3$H]-RU28362 respectively] did not differ significantly from one another. Competitive displacement of [$^3$H]-DEX binding by various steroids revealed a typical GC receptor pattern of relative potencies of displacement; RU 28362 was the most potent displacer, followed by cortisol, progesterone and aldosterone, with estradiol being a poor competitor for [$^3$H]-DEX binding (Fig 2).

To examine whether GC receptors are found in the chromaffin or the non-chromaffin cell populations (or both) present in cultures of adrenal medullary cells, more highly purified chromaffin and non-chromaffin cell cultures were prepared by differential plating (Waymire et al., 1983). Chromaffin-enriched cultures had the highest level of

catecholamines, followed by the standard Percoll-isolated medullary cells, while the non-chromaffin cell cultures had low amounts of catecholamines (Fig. 3b), confirming the separation of cell types. Levels of soluble [$^3$H]-RU28362 binding sites, determined using a saturating concentration of the radioligand, were comparable in all three types of culture - the Percoll-isolated, chromaffin-enriched and non-chromaffin cell populations (Fig. 3a).

**Translocation of GC Receptor and Nuclear Uptake of [$^3$H]-DEX**

Translocation of non-transformed ligand-bound GC receptors from a soluble to an insoluble (chromatin-bound) compartment in response to GCs was estimated by incubating cultures with or without exogenous cortisol and comparing levels of [$^3$H]-DEX binding sites in soluble fractions subsequently prepared from the cells; a loss of soluble sites may be assumed to be due to a translocation of the occupied sites to an insoluble state. Medullary cultures incubated with 50 nM cortisol for 45 min showed a 41% decrease in soluble [$^3$H]-DEX binding sites immediately following removal of cortisol (time 0, Fig. 4a). By 30 minutes following cortisol removal, soluble GC receptor levels returned to control values and remained there for up to 6 h.

The decrease in soluble [$^3$H]-DEX binding sites immediately following a 45 min exposure to different concentrations of cortisol was next examined (Fig. 4b). Significant ($p<0.05$) loss of soluble receptors was found at all concentrations ($5\times10^{-8}$ - $10^{-4}$ M) of cortisol tested except $10^{-8}$ M. The magnitude of the loss in soluble sites was approximately equivalent for cortisol concentrations from $5\times10^{-8}$ to $10^{-6}$ M. A concentration of $10^{-5}$ M cortisol produced a significantly ($p<0.05$) greater loss in soluble sites compared to $10^{-6}$ M, and the highest concentration of cortisol tested ($10^{-4}$ M) produced a greater ($p<0.05$) loss in soluble sites, compared to all other concentrations tested. To ensure that the loss in [$^3$H]-DEX binding sites was due to translocation and not to residual cortisol in the soluble fraction interfering with the binding of ligand to

receptor, in one set of experiments cell cultures were incubated with the highest concentration ($10^{-4}$ M) of cortisol used and subsequently prepared soluble fractions were stripped of free cortisol (using Sephadex LH-20 and verified by radioimmunoassay). In stripped compared to non-stripped soluble fractions, the loss of [$^3$H]-DEX binding sites in response to $10^{-4}$ M cortisol was identical, indicating that residual cortisol does not interfere with measurement of these sites.

To verify the apparent translocation of GC receptors from a soluble to an insoluble or chromatin-bound compartment, nuclear uptake of [$^3$H]-DEX was examined by incubating intact cultures with [$^3$H]-DEX and determining the amount of [$^3$H]-DEX concentrated in a nuclear fraction subsequently prepared from these cells. Fig. 5 shows that specific nuclear uptake of [$^3$H]-DEX was found at all concentrations tested and that this was concentration dependent.

**PNMT Activity**

In confirmation of previous reports (Hersey and diStefano, 1979; Kelner and Pollard, 1985), continual exposure of medullary cultures to cortisol ($10^{-8}$ to $10^{-4}$ M) for 3 days significantly elevated PNMT activity above levels found in control untreated cultures (Fig. 6). Exposure of these cells to $10^{-6}$ M cortisone, a steroid which does not bind the GC receptor, for 3 days had no effect on PNMT activity (data not shown). Of note in the present study is the observation that the magnitude of PNMT elevation was approximately equivalent for cortisol concentrations from $10^{-8}$ to $10^{-5}$ M, while $10^{-4}$ M cortisol produced a further increase in PNMT; this pattern of response to increasing concentrations of cortisol was similar to that seen for translocation of [$^3$H]-DEX binding sites (Fig. 4b).

To test whether increases in PNMT activity induced by both lower and higher concentrations of cortisol were GC receptor-mediated, the ability of the GC receptor antagonist RU 38486 to block cortisol-induced changes in PNMT activity was examined

(Table 1). RU 38486 effectively reversed cortisol-induced elevations in PNMT activity at $10^{-7}$ M and $10^{-5}$ M (and to some extent $5x10^{-5}$ M) cortisol, without affecting PNMT activity on its own. (Note that in experiments with $10^{-7}$ M cortisol, RU 38486 was used at a 10-fold higher concentration ($10^{-6}$ M) than cortisol; in experiments with $10^{-5}$ or $5x10^{-5}$ M cortisol, RU 38486 was used at concentrations of $10^{-5}$ and $5x10^{-5}$ M since cell toxicity of RU 38486 precluded use of higher concentrations of the antagonist).

In order to more closely mimic the short pulse of GCs that occurs with a physiological stressor, medullary cultures were exposed to a 2h pulse of either $10^{-5}$ or $10^{-4}$ M cortisol, the cortisol was removed and PNMT activity measured 3 days later. Fig. 7 shows that at both concentrations tested, a 2h pulse of cortisol significantly elevated PNMT activity assayed 3 days later, when compared to control untreated cultures.

## Discussion

The present study confirms the presence of soluble high affinity [$^3$H]-DEX binding sites in bovine adrenal medullary cells. The $K_d$ (3.3 nM) for [$^3$H]-DEX binding was similar to that reported by Kelner and Pollard (1985) (1.28 nM) but lower than that reported by Nawata and coworkers (1985) (35 nM), using a similar bovine medullary preparation. Nawata et al. (1985), in measuring GC receptors, did not include molybdate in their buffers, a condition which is mandatory for the effective stabilization of GC (type II) receptor binding (Kalimi and Hubbard, 1983; Mitchell et al., 1986). Although [$^3$H]-DEX binds to both mineralocorticoid (type I) and GC corticosteroid receptors (Luttge et al., 1989), the profile of displacement by various steroids indicates that the radioligand binds mainly to GC receptors in adrenal medullary cells. There was no significant difference in the Bmax for [$^3$H]-DEX and for [$^3$H]-RU 28362, a specific GC receptor ligand (Philibert and Moguilewsky, 1983; Coirini et al., 1985), and Scatchard analysis of

saturation experiments revealed only a single population of [$^3$H]-DEX binding sites, further supporting [$^3$H]-DEX binding to mainly GC receptors.

There is, nonetheless, some suggestion that a very small proportion of [$^3$H]-DEX binding sites, just at the limit of the level of detection, may represent binding to a site other than GC receptors. This is suggested by the observations that a small amount of [$^3$H]-DEX binding could not be displaced by high concentrations of RU 28362 (a compound not previously tested as competitor for [$^3$H]-DEX binding in medullary cells). Secondly, the average Bmax for [$^3$H]-DEX tended to be slightly higher (although not significantly) than that for [$^3$H]-RU 28362. This is consistent with findings that [$^3$H]-DEX appears to bind to a small sub-population of mineralocorticoid receptors in brain (see Luttge et al., 1989). However, we have assessed binding of both [$^3$H]-cortisol (in the presence of RU 28362 sufficient to block binding to the GC receptor) and [$^3$H]-aldosterone and have been unable to measure mineralocorticoid receptors in adrenal medullary cells.

The results of experiments with chromaffin and non-chromaffin cell populations enriched by differential plating indicate that GC receptors are present at very similar levels in both chromaffin and non-chromaffin cells in medullary cultures. This finding is not surprising since GC receptors have been found in all nucleated cell types tested. Strong evidence for the presence of GC receptors in adrenal medullary cells has been provided by recent reports of the immunocytochemical localization of the GC receptor in PNMT immunoreactive cells in perfused rat adrenal slices (Ceccatelli et al., 1989) and the identification of a functional GC response element on the rat PNMT gene (Ross et al., 1990), as well as consensus sequences for GC response elements in bovine and human PNMT genes (Baetge et al., 1988; Batter et al., 1988). The presence of the GC receptor in both chromaffin and non-chromaffin cells in medullary cultures should be kept in mind when using these cells as a model for GC regulation of neuroendocrine function. It will

obviously be important to be aware of both purity and composition of cultures when interpreting studies which measure GC receptors and their actions in this preparation.

Basal plasma levels of GCs reaching most tissues (other than the adrenal) are in the range of $10^{-8}$ - $10^{-7}$ M, and these may rise to $10^{-6}$ M during a stressful event (Zumoff et al., 1974; Schoneshofer and Wagner, 1977; Dallman et al., 1987). In many of these tissues, nM concentrations of GCs have been shown to translocate GC receptors (see McEwen et al., 1986). By contrast, levels of GCs measured in the adrenal vein of the anesthetized rat have been measured to be between 6 x $10^{-5}$ M and $10^{-4}$ M (Jones et al., 1977), indicating that the adrenal medulla will be exposed to concentrations of GCs at least as high as $10^{-4}$ M during a stressful event. Given these high levels of GCs in the adrenal medulla, it was unclear whether GC receptors in this tissue would respond to low concentrations of GCs as do other tissue types. Our results indicate that there is significant translocation of [$^3$H]-DEX binding sites in adrenal medullary cells in response to exogenous GC levels as low as 50 nM. In a similar low concentration range (5-100 nM), nuclear uptake of [$^3$H]-DEX was found. (Note that cortisol was used to induce translocation of GC receptors whereas [$^3$H]-DEX was used for the nuclear uptake and that data for the two measures are expressed in different units. Thus quantitative comparison between the absolute amounts of GC receptor translocated and nuclear uptake of [$^3$H]-DEX should not be made.) The loss in soluble binding sites in response to a 45 minute cortisol exposure suggests a translocation, and not a downregulation, of the GC receptor, since downregulation of GC receptors occurs with a much slower time course (approximately 24h GC exposure, see Svec 1985 for a review). Following removal of cortisol, soluble receptor levels return to control values within 30 minutes, suggesting a possible reconstitution of recycled GC receptors (Raaka and Samuels, 1983; Munck and Holbrook, 1984) able to rebind ligand. Translocation of [$^3$H]-DEX binding sites appears to plateau with exposure to increasing concentrations of GC from 5x$10^{-8}$ to $10^{-6}$ M. However, at $10^{-5}$ and $10^{-4}$ M cortisol, there is a further significant increase in the

magnitude of translocation. Thus it appears that [$^3$H]-DEX binding sites in adrenal medullary cells do respond to nM concentrations of GCs, but that an additional population of sites is translocated in response to very high concentrations of GCs physiologically seen by the medulla. In striking correlation with this was our observation that the increase in PNMT following 3 day exposure to cortisol also reached a plateau between $10^{-8}$ and approximately $10^{-5}$ M cortisol, with a further significant increase in PNMT levels evident at $10^{-4}$ M. Thus the results show that GC receptors can be translocated and PNMT activity can be increased by high concentrations of cortisol, over and above the response observed when lower cortisol concentrations are used. With respect to physiological mechanisms it is tempting to speculate that the plateau responses to lower cortisol concentrations might represent permissive effects of the GCs under basal conditions while the enhanced effect at high cortisol concentrations relates to conditions of augmented GC release such as occurs during stress.

It is not clear what mechanism(s) may underly the very distinctive biphasic effect of low and high concentrations of cortisol on PNMT induction (in addition to translocation of [$^3$H]-DEX binding sites). To date, there are no reports of separate high and low affinity GC receptor populations as might be suggested by these findings. The observation that effects of both low ($10^{-7}$ M) and high ($10^{-5}$, $5x10^{-5}$ M) concentrations of cortisol on PNMT are blocked by RU 38486 is consistent with both low and high cortisol concentrations modulating PNMT activity through interactions with a GC receptor. However, it should be noted that RU 38486 is not GC receptor specific (see Philibert et al., 1985), thus this experiment cannot completely rule out the possibilty that steroid receptors other than the GC receptor (e.g. progesterone receptors) may mediate effects of low or high cortisol on PNMT. (To our knowledge, the presence of steroid receptors other than the GC receptor, in the adrenal medulla has not been investigated). However, Hersey and diStefano (1979) have shown that progesterone is ineffective in inducing PNMT activity in adrenal medullary cells in culture. An alternative possibility to

explain differential effects of low and high cortisol concentrations is that a metabolic mechanism regulating cortisol levels seen by the receptor saturates at high cortisol concentrations, thus allowing a larger proportion of free cortisol to act on the receptor population.

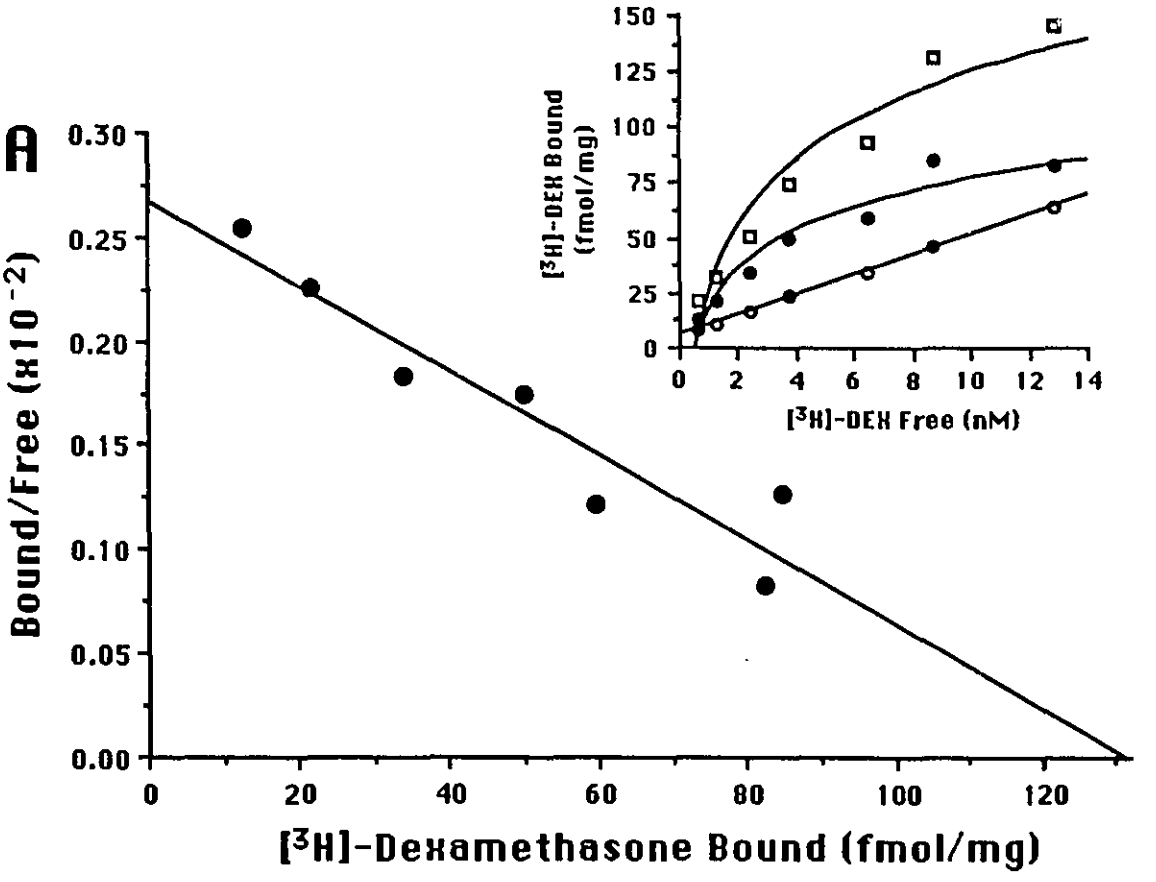
Short-term cortisol exposure is sufficient to mediate a GC receptor signal, as demonstrated by both the loss of soluble binding sites and the nuclear uptake of [$^3$H]-DEX. Thus, it seemed plausible that GC regulation of PNMT activity should occur in response to these short-term dynamic changes in the hormone-receptor signal. However, previous studies showing GC regulation of catecholamine synthesis in adrenal medullary cells indicated that 18h to 2 days of continual exposure to GCs is necessary to produce increases in PNMT activity or catecholamine levels and that shorter times are ineffective (Hersey and diStefano, 1979; Kelner and Pollard, 1985; Nawata et al., 1985). Since an acute stress response results in an increase in GC release lasting an hour or two, the importance of acute stress-induced increases in released GC on modulation of adrenal catecholamines was questioned. No previous experiments have exposed adrenal medullary cells to a short pulse of GCs and measured PNMT activity after a lag period of several days following GC exposure. The present study shows that a 2h pulse of cortisol is able to elevate adrenal chromaffin cell PNMT, if PNMT levels are measured 3 days following cortisol exposure. Bohn and colleagues (1984) have reported similar findings in another catecholaminergic preparation, the rat superior cervical ganglion, where a short (2 to 4 h) pulse of $10^{-6}$ M DEX elevates PNMT measured 2 days after DEX exposure. It should be noted that in the present experiments, adrenal medullary cultures were maintained without any added GC before the 2h GC exposure in order to maximize the possibility of observing GC effects with this short GC exposure. This contrasts with the *in vivo* situation where a high basal adrenal cortisol level is raised to an even higher level by a stressor. Nevertheless, recent *in vivo* experiments in our lab show that 20 minute restraint stress in rats raises adrenal PNMT levels measured 24h (but not earlier)

following the stress (K. Betito, J.B. Mitchell, S. Bhatnagar, M.J. Meaney, and P. Boksa, in preparation) indicating that the time course of events we have observed in the cell culture system may well reflect the *in vivo* situation.

Further unpublished observations from our laboratory indicate that a lag period of at least 24-48h after a 2h pulse of GCs is necessary in order to observe an increase in PNMT activity in chromaffin cell cultures. Thus, our results suggest that a short-term stress-induced elevation in GC release could regulate adrenal PNMT, with elevations in the enzyme activity occurring after a necessary lag period. If such were the case, GCs at the level of the adrenal medulla would appear to play a role not in the initial stress response per se but rather in the recovery of the animal from stress (i.e. promoting replenishment of catecholamine levels depleted during stress). In this context, Munck (1971) has previously suggested that many well-known GC effects (e.g. their anti-inflammatory, immunosuppressive, and insulin-reducing effects) can be interpreted as functioning to protect the organism against prolonged stress-induced defense reactions thus aiding in the return to homeostatic conditions. There is evidence for at least two mechanisms by which GCs induce PNMT activity in adrenal chromaffin cells; both of these mechanisms require a fair amount of time and could account for the lag period necessary to induce PNMT even after a short pulse of GCs. Earlier studies suggesting that GCs increase the rate of PNMT synthesis (Wurtman and Axelrod, 1966; Ciaranello, 1978) are strongly supported by more recent experiments showing that GCs increase levels of mRNA for adrenal PNMT in vivo (Stachowiak et al., 1988) and in vitro, where this effect is blocked by the GC receptor antagonist RU 38486 (Wan and Livett, 1989), and by the demonstration of a functional GC response element in the rat PNMT promoter (Ross et al., 1990). On the other hand, there is also evidence that GCs can decrease the rate of PNMT degradation (Ciaranello, 1978) probably through regulation of the metabolism of S-adenosyl methionine, the methyl donor for PNMT (Berenbeim et al., 1979; Wong et al., 1985).

In summary, the present study shows that bovine adrenal medullary cells contain classical GC receptors, which are translocated in response to nM concentrations of GCs, as has been found in other tissues. Further translocation occurs in response to higher GC concentrations, such as may be encountered in the adrenal during stress, and this is accompanied by further increases in PNMT activity at these high GC levels. This additional translocation of GC receptors at high GC concentrations has not been reported for other tissues, and may be specific for adrenal medullary cells. Adrenal medullary cells respond to a short pulse of GCs, mimicking an acute stress, by increasing PNMT activity 3 days later. Therefore, our results provide evidence that GCs in the adrenal medulla may play a more prominent role in the stress response, and particularly in the recovery of the organism from a stressful event, than was previously thought. In addition, these studies support the use of bovine adrenal medulary cells in culture as a useful model to study GC regulation of neuroendocrine function.

**Figure II-1. Saturation analyses of [$^3$H]-dexamethasone (A) and [$^3$H]-RU 28362 (B) binding to cytosolic fractions of bovine adrenal medullary cells.** Representative saturation analyses (insets) and corresponding Scatchard analyses are shown.


A
Bound/Free (x10$^{-2}$)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
20
40
60
80
100
120
[$^3$H]-Dexamethasone Bound (fmol/mg)
[$^3$H]-DEX Bound (fmol/mg)
150
125
100
75
50
25
0
0
2
4
6
8
10
12
14
[$^3$H]-DEX Free (nM)

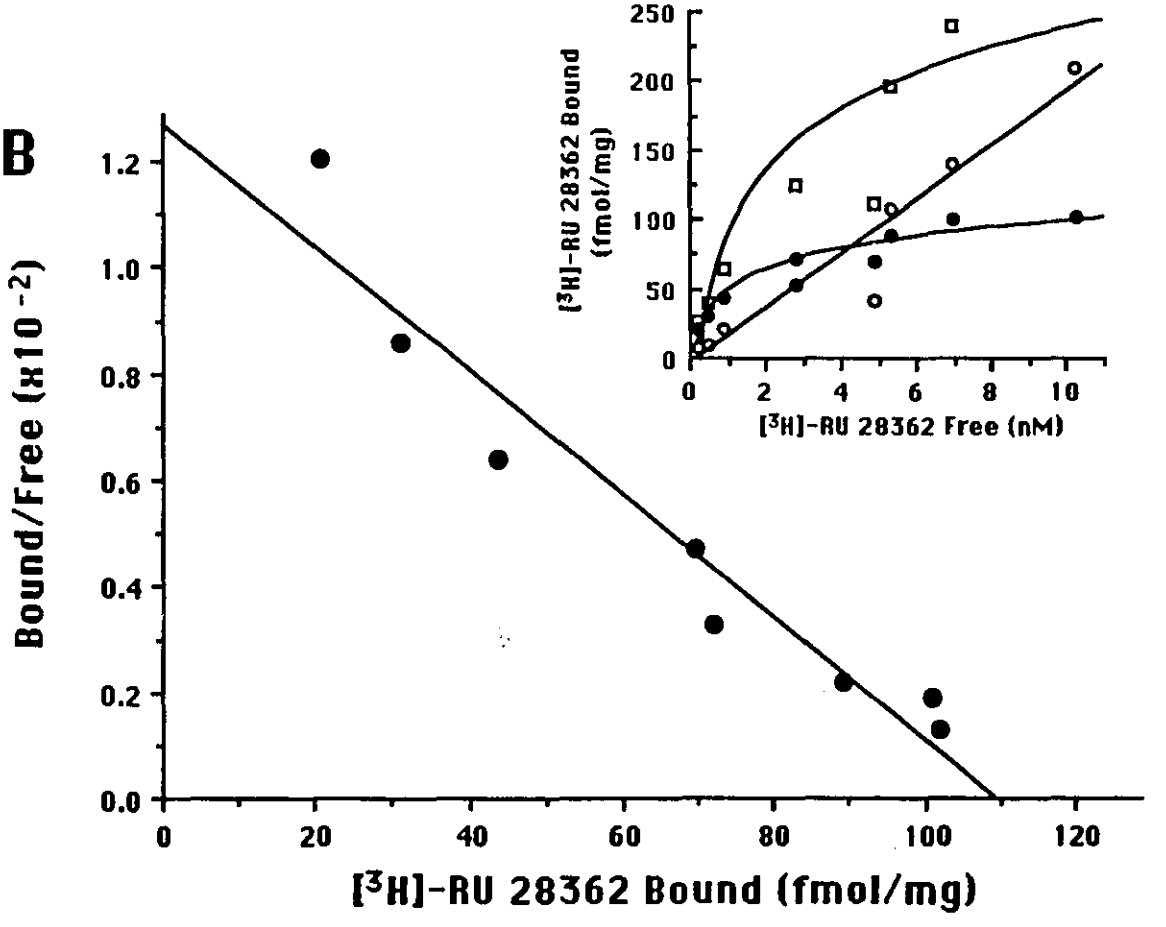

B
Bound/Free (x10$^{-2}$)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
[$^3$H]-RU 28362 Bound (fmol/mg)
[$^3$H]-RU 28362 Bound (fmol/mg)
250
200
150
100
50
0
0
2
4
6
8
10
[$^3$H]-RU 28362 Free (nM)

**Figure II-2. Competition by various steroids for specific binding of [$^3$H]-DEX.** A saturating (12 nM) concentration of [$^3$H]-DEX was competed by various concentrations of steroids. Each point represents the mean of 4-12 determinations except for RU 28362 at $10^{-5}$ M which is the mean of 3 determinations. (Modified from original published figure).

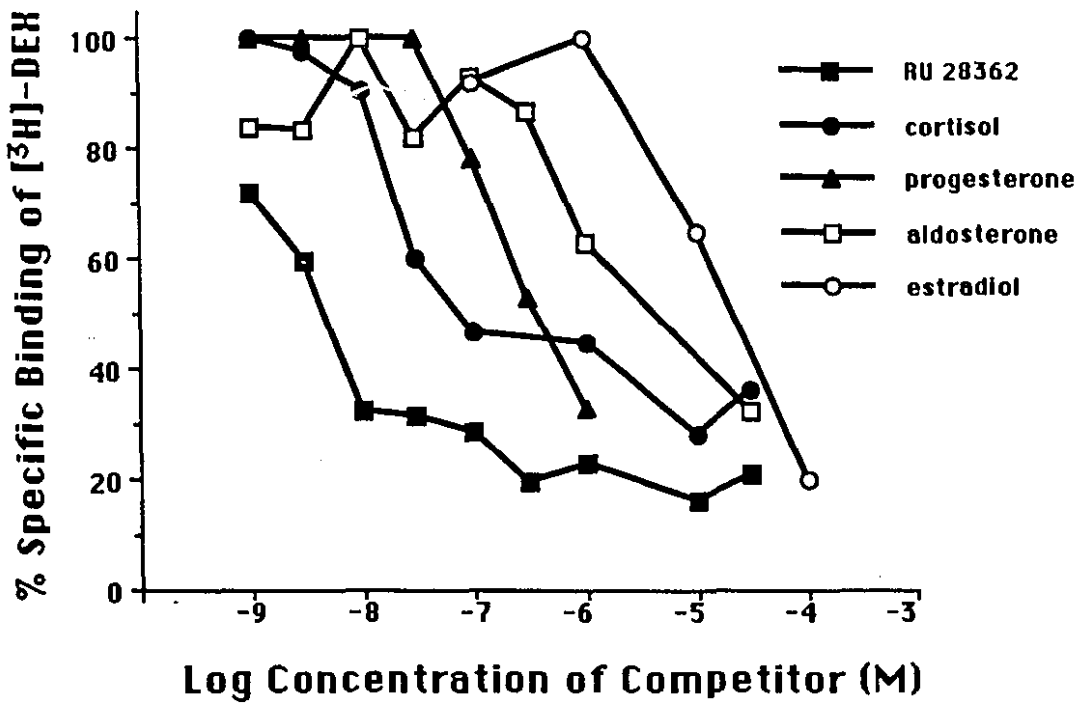
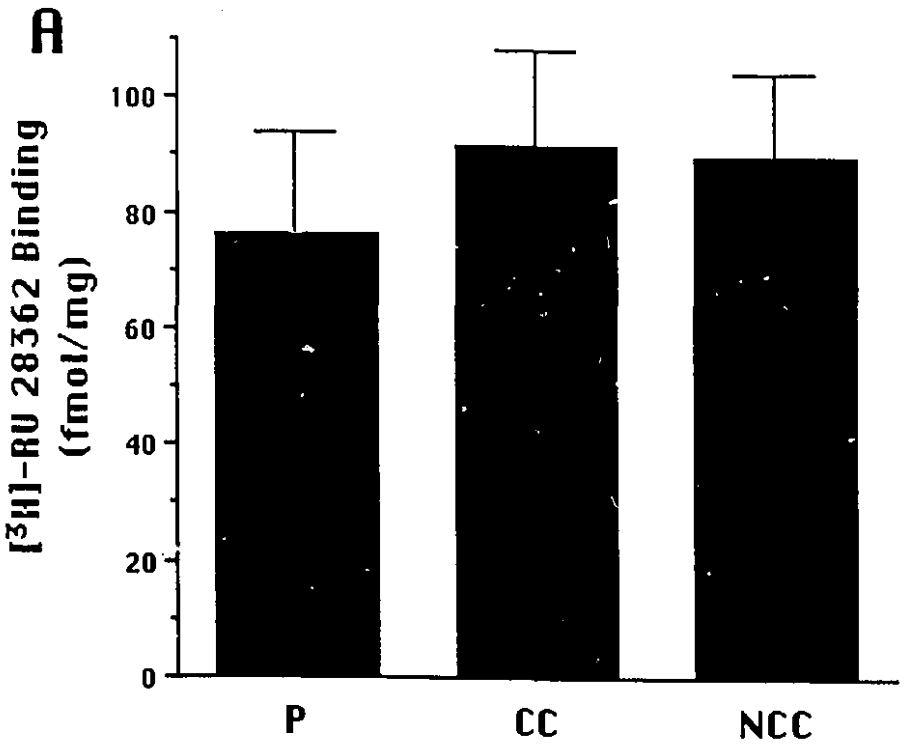

% Specific Binding of [3H]-DEX
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
-3
Log Concentration of Competitor (M)
RU 28362
cortisol
progesterone
aldosterone
estradiol

**Figure II-3. Levels of (A) [$^3$H]-RU 28362 binding sites and (B) catecholamines (CAs) in differentially plated bovine adrenal medullary cells.** P=Percoll isolated, CC=chromaffin cell-enriched, NCC=non-chromaffin cell-enriched preparations. In (A), each bar represents the mean (±SEM) of 6-12 determinations. (Y-axis label modified from original published figure). In (B), each bar represents the mean (±SEM) of 4 determinations in a representative experiment.


A
[3H]-RU 28362 Binding
(fmol/mg)
100
80
60
40
20
0
P
CC
NCC

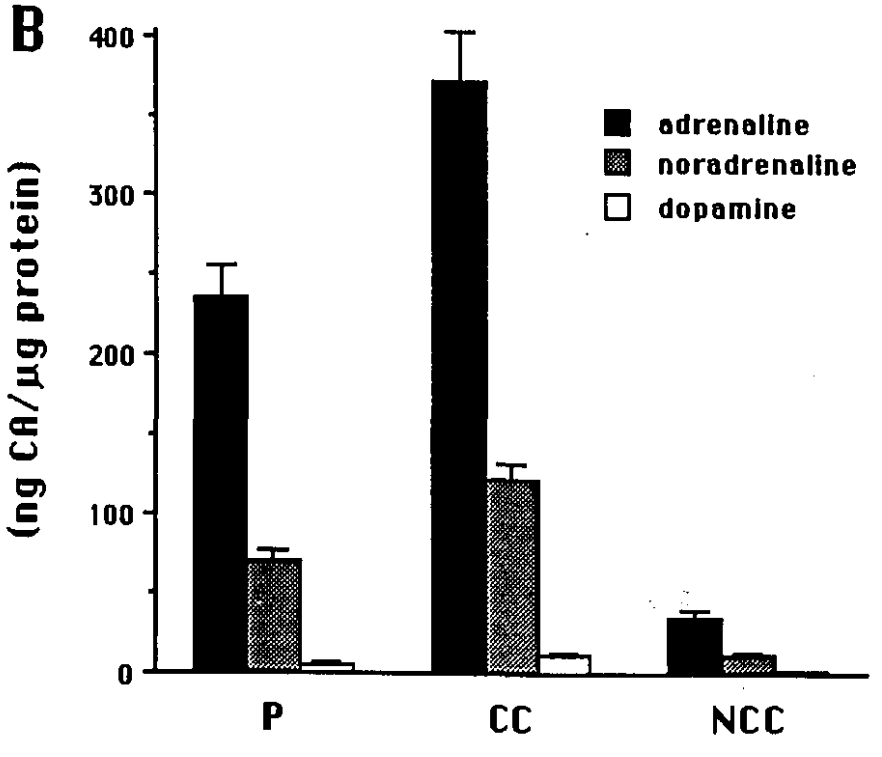
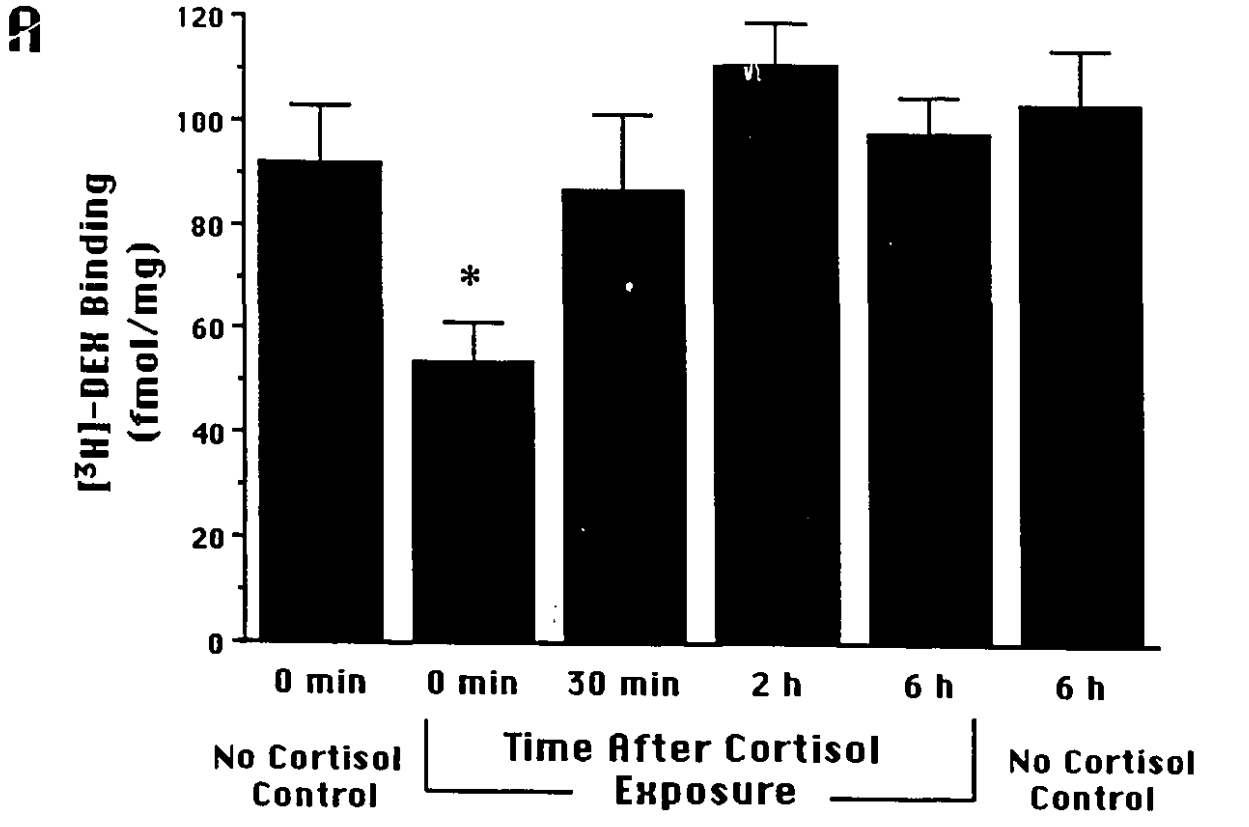

B
(ng CA/µg protein)
400
300
200
100
0
adrenaline
noradrenaline
dopamine
P
CC
NCC

**Figure II-4. Translocation of the GC receptor from a soluble to an insoluble compartment in response to cortisol exposure.** (A) Time course of recovery from cortisol exposure: Adrenal medullary cells were exposed to 50 nM cortisol for 45 minutes, the medium replaced with steroid-free medium and a cytosolic extract was prepared at different time points as indicated in the figure. The levels of soluble [$^3$H]-DEX binding sites were measured as described in Experimental Procedures. Each bar represents the mean (± SEM) of 13-16 single point determinations (* $p<0.05$ versus 0-min control) (B) Cortisol concentration response curve for GC receptor occupancy: Cells were exposed to the indicated concentrations of cortisol for 45 minutes and a soluble extract prepared immediately following exposure. The levels of soluble [$^3$H]-DEX binding sites were measured as described in Experimental Procedures. Each point (open circles) represents the mean (± SEM) of 11-23 single point determinations [* $p<0.02$ versus control (0 cortisol; filled circle), † $p<0.02$ versus $10^{-5}$ M]. The open square at $10^{-4}$ M represents soluble extract that has been stripped of free cortisol using Sephadex LH-20 columns. Both of the points at $10^{-4}$ M are significantly different ($p<0.02$) from all other points on the graph; $10^{-5}$ M is significantly ($p<0.02$) different from both $10^{-6}$ M and $10^{-4}$ M. (Y-axis labels modified from original published figure).


A
120
100
80
60
40
20
0
[3H]-DEX Binding
(fmol/mg)
*
0 min
0 min
30 min
2 h
6 h
6 h
No Cortisol
Control
Time After Cortisol
Exposure
No Cortisol
Control

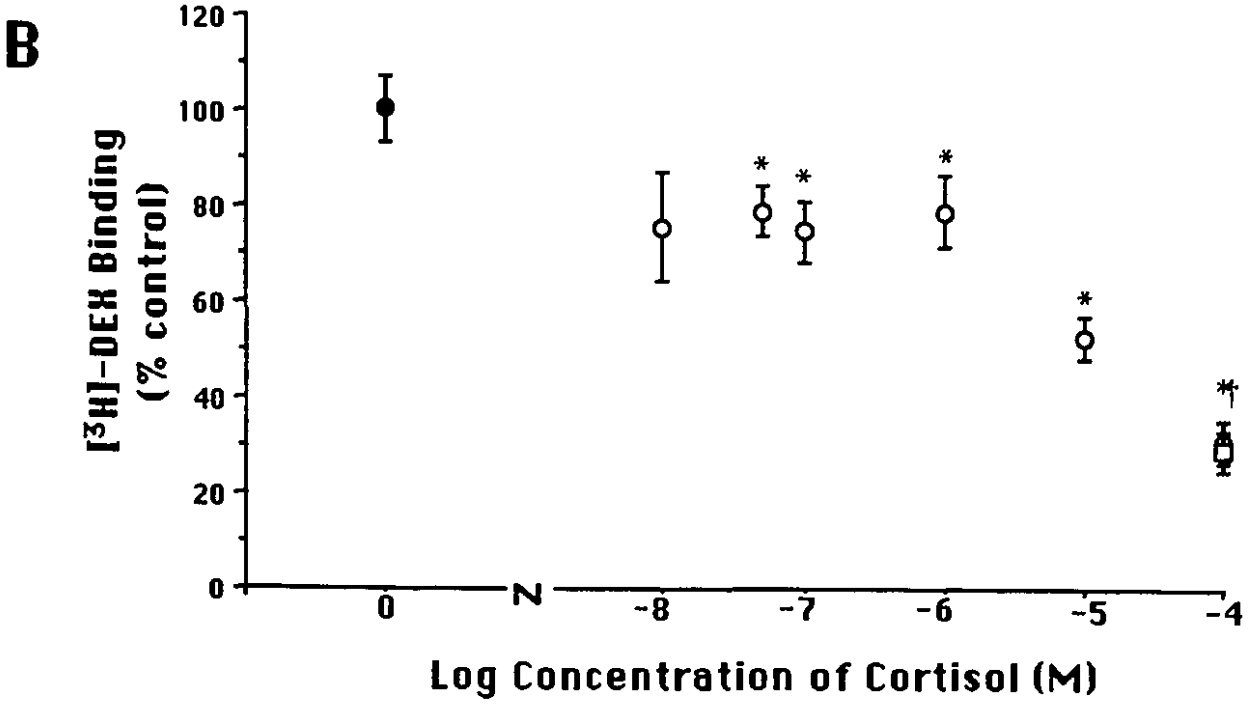

B
120
100
80
60
40
20
0
[3H]-DEX Binding
(% control)
*
0
N
-8
-7
-6
-5
-4
Log Concentration of Cortisol (M)

**Figure II-5. Nuclear uptake of [$^3$H]-DEX.** Adrenal medullary cells were exposed to various concentrations of [$^3$H]-DEX for 45 minutes, and the tritium label quantified in a subsequently prepared nuclear fraction as a measure of nuclear uptake. Each point represents the mean (±SEM) from 3-4 determinations.

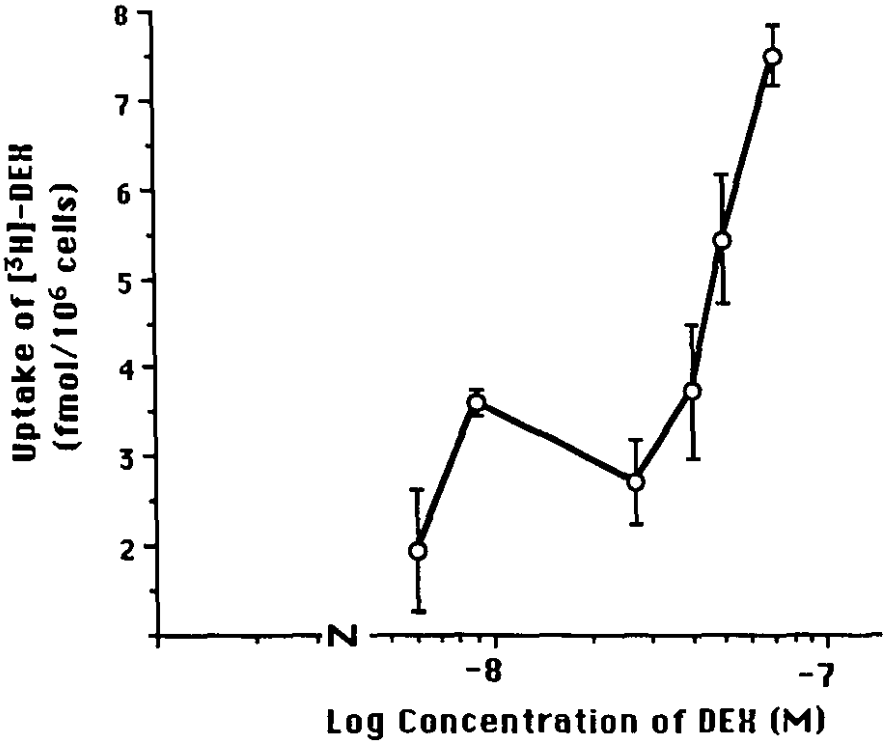

Uptake of [3H]-DEX
(fmol/10^6 cells)
8
7
6
5
4
3
2
N
-8
-7
Log Concentration of DEX (M)

**Figure II-6. PNMT activity in adrenal medullary cells following a 3 day exposure to various concentrations of cortisol.** Cells were maintained in a serum-free medium before treatment as described in Experimental Procedures. Each control point (0 cortisol) represents the mean (±SEM) of 51 determinations and all other points are the mean (±SEM) of 21-30 determinations. [* $p<0.02$, **$p<0.005$, *** $p<0.001$ versus control (filled circle), † $p<0.05$ versus $10^{-5}$ M]

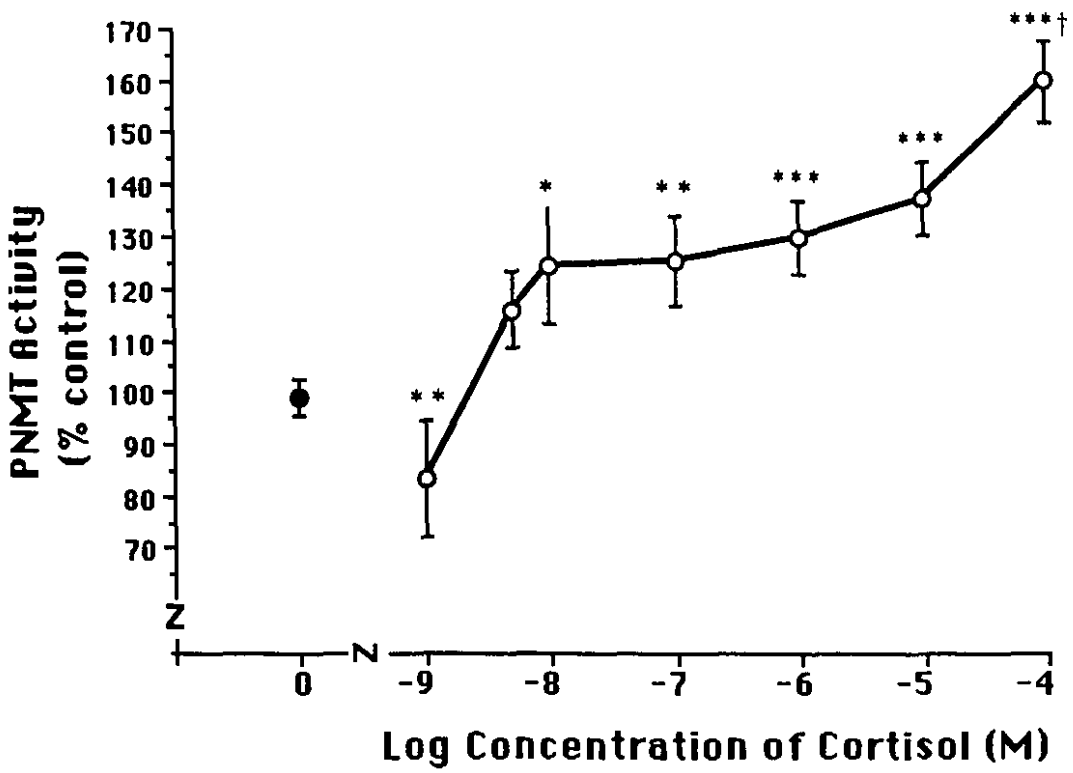

PNMT Activity
(% control)
170
160
150
140
130
120
110
100
90
80
70
Z
N
0
-9
-8
-7
-6
-5
-4
Log Concentration of Cortisol (M)
**
*
**
***
***
***†

**Figure II-7. A short (2h) pulse of cortisol elevates PNMT activity 3 days later.** Adrenal medullary cells were exposed to cortisol for 2 hours, the medium replaced with steroid-free medium, and PNMT activity measured 3 days following the beginning of exposure. Each bar represents the mean (±SEM) of 16-24 determinations. (*** $p<0.001$ versus control).

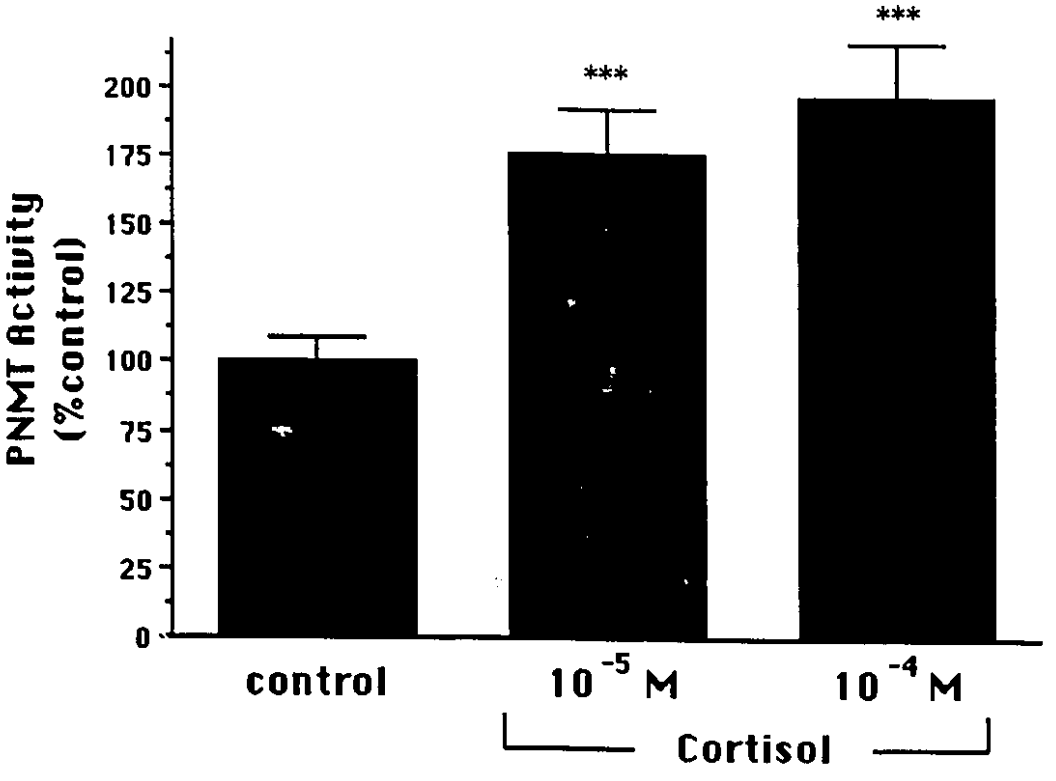

PNMT Activity
(%control)
200
175
150
125
100
75
50
25
0
***
***
control
10 -5 M
10 -4 M
Cortisol

**Table II-1. Effects of the GC receptor antagonist, RU 38486, on cortisol-induced increases in PNMT.**

| Condition | PNMT Activity (% control) |
|---|---|
| Control | 100.0 ± 2.8 (58) |
| Ethanol | 100.6 ± 4.8 (46) |
| Cortisol $10^{-7}$ M | 113.3 ± 3.8 (30)[a] |
| + $10^{-6}$ M RU 38486 | 99.2 ± 4.3 (34)[c] |
| Cortisol $10^{-5}$ M | 288.7 ± 25.4 (10)[b] |
| + $10^{-5}$ M RU 38486 | 125.7 ± 12.9 (13)[d] |
| + $5x10^{-5}$ M RU 38486 | 140.7 ± 20.2 (12)[d] |
| Cortisol $5x10^{-5}$ M | 242.7 ± 12.4 (12)[b] |
| + $10^{-5}$ M RU 38486 | 160.3 ± 14.9 (12)[b,e] |
| + $5x10^{-5}$ M RU 38486 | 181.9 ± 13.6 (8)[b,e] |
| RU 38486 | |
| $10^{-6}$ M | 97.4 ± 4.8 (31) |
| $5x10^{-5}$ M | 99.4 ± 11.5 (16) |

Adrenal medullary cells were maintained in a serum-free medium and subsequently exposed to cortisol in the presence or absence of RU 38486, in the indicated concentrations, for 3 days. Following this, PNMT activity was measured. Results are the mean ± SEM (n).

a = different from control, $p<0.05$
b = different from control, $p<0.0001$
c = different from $10^{-7}$ M cortisol alone, $p<0.05$
d = different from $10^{-5}$ M cortisol alone, $p<0.0001$
e = different from $5x10^{-5}$ M cortisol alone, $p<0.02$

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# Chapter III. Glucocorticoid Receptors in Bovine Adrenal Medullary Cells in Culture: Regulation by Cyclic Nucleotides

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Abbreviations and trivial names used: BSA, bovine serum albumin; cAMP, 3',5'-cyclic adenosine monophosphate; cGMP, 3',5'-cyclic guanosine monophosphate; DEX, dexamethasone; DMEM, Dulbecco's Modified Eagle's Medium; GC, glucocorticoid; PNMT, phenylethanolamine N-methyltransferase; RU 38486, 17ß-hydroxy-11ß,-4-dimethylaminophenyl-17a-propynylestra-4,9-diene-3-one.

## Abstract

Glucocorticoid receptor levels within a given cell determines the glucocorticoid effect in the target tissue. GC receptors are present in adrenal medullary cells in culture where they are involved in the regulation of catecholamine biosynthesis. Modulation of GC receptor protein and/or mRNA levels in response to cyclic nucleotides has been found in various cells types. In this study, we have investigated the effects of cAMP and cGMP on GC receptor binding and GC receptor-mediated function in Percoll-isolated bovine adrenal medullary cells in culture. Four day treatment of cells with 8 bromo-cAMP (8 Br-cAMP; $10^{-3}$ M) an analogue of cAMP, or forskolin ($10^{-5}$ M) an activator of adenylate cyclase, decreased soluble [$^3$H]-dexamethasone (DEX) binding by 55% and 54%, respectively. 8 bromo-cGMP (8 Br-cGMP) treatment decreased [$^3$H]-DEX binding by 31 and 34% at $10^{-5}$ M and $10^{-4}$ M respectively. Treatment with 8 Br-cAMP or forskolin, but not 8 Br-cGMP, elevated cortisol levels in the medium of treated cells, presumably by elevating steroidogenesis in contaminating cortical cells. Cultures further purified to produce chromaffin-enriched cell cultures, also showed a loss (41%) in soluble [$^3$H]-DEX binding when treated with 8 Br-cAMP ($10^{-3}$ M). Four day treatment of standard Percoll-isolated cells with low concentrations of cortisol ($10^{-9}$ M to $2x10^{-7}$ M) similar to that found in the medium of 8 Br-cAMP treated cells, did not decrease soluble [$^3$H]-DEX binding, whereas higher cortisol concentrations ($10^{-6}$ M) produced a 62% loss in soluble binding. Adsorption of cortisol with bovine serum albumin (5 mg/mL) prevented a cortisol ($10^{-6}$ M)-induced loss in soluble [$^3$H]-DEX binding with no effect on the 8 Br-cAMP-induced loss in binding, suggesting that the decrease in binding observed following 8 Br-cAMP treatment is not due to the release of cortisol from contaminating cortical cells. Finally, we report a loss in the ability of 8 Br-cAMP or -cGMP treated cells to fully induce the activity of phenylethanolamine N-methyltransferase (PNMT) in response to cortisol, indicating that decreases in soluble [$^3$H]-DEX binding

translate into a decrease in the functional consequence of GC receptor binding in adrenal medullary cells.

In conclusion, these results indicate that long-term increases in cyclic nucleotide second messengers are able to decrease GC receptor binding in bovine adrenal medullary cells, via a mechanism independent of released cortisol. The study provides the first report that alterations in GC receptor levels are reflected in an alteration in a GC-mediated function, i.e. induction of PNMT, in the adrenal medulla. It is suggested that the pattern and timing of exposure of adrenal medullary cells to GCs and cyclic nucleotides may have an important influence on the cellular response to these agents.

## Introduction

In a variety of cell types, the number of glucocorticoid (GC) receptor molecules contained in the cell has been shown to determine the magnitude of the biological response to the GCs (Bourgeois and Newby, 1979; Vanderbilt et al., 1987; Distelhorst, 1989; Dong et al., 1990; Tanaka et al., 1992). In several tissues, GCs themselves have been shown to regulate GC receptor and/or mRNA levels (Svec and Rudis, 1981; Sapolsky et al., 1984; McIntyre and Samuels, 1985; Svec, 1985a; Svec, 1985b; Okret et al., 1986; Kalinyak et al., 1987; Miller et al., 1990; Govindan et al., 1991; O'Donnell and Meaney, 1991). Other factors, such as various second messengers, have also been considered for their ability to regulate GC receptor and/or mRNA levels. Modulation of the GC receptor system in response to cyclic nucleotides has been found in rat hippocampal cells (Mitchell et al., 1992), human skin fibroblasts (Oikarinen et al., 1984) and mononuclear leukocytes (Tanaka et al., 1992), murine lymphoma cells (Gruol et al., 1986; Gruol et al., 1989), rat HTC cells (Dong et al., 1989; Okret et al., 1991), and AtT20 cells (Sheppard et al., 1991) in culture. In all but the AtT20 cell line, cAMP increased GC receptor binding and/or mRNA. Conversely, in fibroblasts, cyclic GMP decreased GC receptor binding, raising the possibility that the cAMP to cGMP ratio may regulate GC receptor levels within a cell (Oikarinen et al., 1984).

Several groups (Kelner and Pollard, 1985; Nawata et al., 1985; Betito et al., 1992) have demonstrated the presence of GC (type II corticosteroid) receptors in bovine adrenal medullary cell cultures. To date, regulation of GC receptors by neurotransmitter second messengers has not been studied in the adrenal medulla. In bovine adrenal medullary cells in culture, formation of cAMP occurs in response to activation of receptors for a variety of neurotransmitters and neuromodulators including ACh at nicotinic receptors (Guidotti and Costa, 1974; Eiden et al., 1984b), corticotropin releasing factor (CRF; Udelsman et al., 1986), vasoactive intestinal peptide (VIP;

Wilson, 1988), adrenocorticotropic hormone (ACTH; Michener et al., 1985), and histamine (Marley et al., 1991). Formation of cGMP occurs in response to muscarinic ACh stimulation (Yanagihara et al., 1979; Schneider et al., 1979; Kayaalp and Neff, 1979), to imidazole receptor activation following clonidine treatment (Regunathan et al., 1990; Regunathan et al., 1991), and to atrial natriuretic peptide (ANP; Heisler and Morrier, 1988). In the adrenal medulla, as in many target tissues, the effects of both GCs and cAMP are very similar. It has been reported that both cAMP analogues (or elevation of endogenous cAMP), and GCs can elevate activity and/or mRNA of adrenomedullary tyrosine hydroxylase (TH), the rate limiting enzyme in catecholamine biosynthesis (Levitt et al., 1965), and of phenylethanolamine N-methyltransferase (PNMT), the enzyme catalysing the conversion of noradrenaline to adrenaline (Axelrod, 1962), in a variety of in vitro and in vivo adrenal models (Wurtman and Axelrod, 1965, 1966; Mueller et al., 1970; Weinshilboum and Axelrod, 1970; Ciaranello and Black, 1971; Gewirtz et al., 1971; Pohorecky and Wurtman, 1971; Guidotti and Costa, 1973; Ciaranello et al., 1975; Kurosawa et al., 1976; Hersey and DiStefano, 1979; Kelner and Pollard, 1985; Stachowiak et al., 1988; Wan and Livett, 1989; Ross et al., 1990; Stachowiak et al., 1990a; Stachowiak et al., 1990b; Betito et al., 1992). In addition, both cAMP elevation and GC exposure increase chromaffin cell enkephalin and/or proenkephalin mRNA levels (Eiden and Hotchkiss, 1983; Eiden et al., 1984a; Quach et al., 1984; Yanase et al., 1984; LaGamma and Adler, 1987; Inturrisi et al., 1988; Stachowiak et al., 1990a; Wan et al., 1991). Therefore, both cAMP and GCs have similar regulatory effects on catecholamine biosynthesis and peptide levels in adrenomedullary cells. This raises the possibility that some of the effects of cAMP in the adrenal medulla could be mediated indirectly via cAMP regulation of GC receptors. In the present study, using bovine adrenal medullary cells, we have examined the regulation of GC receptor levels, as measured by soluble [$^3$H]-dexamethasone (DEX) binding, by analogues of the cyclic nucleotide second

messengers, cAMP and cGMP [8 bromo-cAMP (8 Br-cAMP) and 8 bromo-cGMP (8 Br-cGMP)]. Additionally, we have investigated the functional consequence of changing receptor levels by measuring the inducibility of the GC-regulated enzyme, PNMT.

## Experimental Procedures

### Cell Culture

Adrenal medullary cells were isolated by retrograde perfusion of bovine adrenal glands with collagenase and DNase I, and purification of isolated cells on Percoll density gradients as described by Livett (1984). Cells were plated on collagen-coated 35 $mm^2$ culture plates ($10^6$ cells/plate) or 60 $mm^2$ culture plates ($2.5x10^6$ cells/plate) and maintained at 37°C in 5% $CO_2$ in air in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Burlington, Ontario) supplemented with 10% fetal calf serum, 1% glucose, 100 µg/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL gentamycin sulfate, 2.5 µg/mL amphotericin B (Fungizone, GIBCO) and $10^{-5}$ M cytarabine (Cytosar, Sigma, St. Louis, MO; used to prevent growth of rapidly dividing non-chromaffin cells). For all studies, the cells were used between day 6-10 of culture and were placed in serum-free, steroid-free medium [50% DMEM, 50% Ham's F12 Nutrient Mixture (GIBCO)] with antibiotics and Cytosar for at least 2 days before use. Cortisol (Aldrich, Milwaukee, WI), RU 38486 (generously provided by D. Philibert, Roussel-Uclaf, Romainville, France) and forskolin (Sigma) were dissolved in ethanol, and the cyclic nucleotide analogues (8 bromo-cAMP and -cGMP, Sigma) dissolved in water, and appropriate vehicle controls performed with all experiments.

Published data on the purity of medullary cell cultures prepared by protocols similar to the above indicate that such cultures contain approximately 85-90% chromaffin cells and 10-15% non-chromaffin cells (cortical and endothelial cells, fibroblasts, Unsicker and Müller, 1981; Banerjee et al., 1985), with our estimates of purity being

slightly higher (2-5% non-chromaffin cells at time of plating; unpublished data). In one set of studies, isolated medullary cells were further purified into highly chromaffin-enriched and non-chromaffin-enriched cell populations by differential plating according to the method of Waymire et al. (1983). Isolated medullary cells were placed into 80 $cm^2$ plastic flasks (20 x $10^6$ cells/25 mL of serum-free medium without Cytosar/flask). The flasks were incubated at 37°C for 3 hours, and the chromaffin cells were decanted and plated as above.

**GC receptor binding experiments**

Adrenal medullary cells plated in 60 $mm^2$ culture dishes and treated as described in the figure legends, were washed with an ice-cold isotonic salt solution (wash buffer, 154 mM NaCl, 5.6 mM KCl, 10mM glucose, 5 mM trizma acetate; pH=7.5), scraped into ice-cold buffer [TEDGM=30 mM Tris HCl, 1 mM disodium EDTA, 1 mM dithiothreitol, 10% v/v glycerol and 10mM sodium molybdate, pH=7.4], at a concentration of 2.5 x $10^6$ cells/400 µL TEDGM and sonicated on ice. The homogenate was centrifuged at 0-2°C for 45 min at 105 000*g*. GC receptor binding was carried out using methods established by Kalimi and Hubbard (1983). An aliquot (150 µL) of the supernatant (cytosol extract) was incubated with 100 µL aliquots of TEDGM containing [1,2,4 - $^3$H]-DEX (specific activity = 98.2 Ci/mmole; Amersham, Oakville, Ontario) in a final saturating concentration of 10 nM at 0-4°C. Incubation was for 20-22h. Following the incubation, bound steroid was separated from free on Sephadex LH20 (Pharmacia Fine Chemicals, Dorval, Qué.) columns (7 x 1 cm, equilibrated with TEDGM) . Aliquots (100 µL) of the incubates were washed into the columns with 100 µL TEDGM and bound steroid was eluted with 500 µL TEGM (TEDGM without dithiothreitol) 30 minutes later. Radioactivity in the eluate was counted in 4.5 mL Ecolite (ICN, Montreal, Qué.) at 50% efficiency. Non-specific binding was determined using parallel incubations

with a 500-fold excess of unlabelled cortisol (the major endogenous bovine GC). A value for [$^3$H]-DEX binding in a single plate was determined by assaying duplicate aliquots of cytosol derived from that plate and using the mean specific binding obtained as an n of 1. The level of GC receptors between preparations varied between ≈ 30 and 120 femtomoles [$^3$H]-DEX bound per milligram of soluble protein. Given the variability in basal [$^3$H]-DEX binding between cell culture preparations, cells from the same preparation were used for both control and treatment conditions for each experiment, and the data are presented as a percentage of the binding found in control untreated plates within the same preparation. Each treatment was tested using cells from more than one preparation (usually 3). Protein was measured by the method of Bradford (1976). No changes in protein levels were found following any drug treatment. In one set of studies (Table III-5) binding data are expressed as femtomoles of specific [$^3$H]-DEX bound per million cells in order to compare between the various treatment groups since the presence of bovine serum albumin in some of the treated samples prevented the use of a standard protein assay.

## Measurement of Cortisol Concentration

The concentration of cortisol was measured in the medium of adrenal medullary cells following various treatments. An aliquot (250 µL) of the medium was extracted into 750 µL of absolute ethanol (or 10 - 100 µL sample plus ethanol to equal 1 mL in order to dilute samples with expected higher levels of cortisol), the samples centrifuged at 1800*g*, and duplicate 250 µL aliquots of the supernatant were freeze-dried and used for radioimmunoassay of cortisol as previously described (Krey et al., 1975). Control experiments verified that the medium, as well as the cyclic nucleotides, did not interfere with the radioimmunoassay.

### Measurement of cAMP

Cellular cAMP levels were determined using a competitive protein binding technique, based on the competition between radiolabelled cAMP and cAMP levels in the sample, for a protein with high specificity for cAMP (Brown et al., 1971). Cells were scraped into ethanol, allowed to stand 5 min at room temperature to denature proteins, centrifuged and processed for use in the cAMP assay kit (Amersham, Arlington Heights, IL) as described in detail elsewhere (Mitchell et al., 1992). Control levels of cAMP ranged from 200-600 pmol/mg protein.

### PNMT activity

Following treatment, adrenal medullary cells plated in 35 $mm^2$ culture dishes were scraped into 500 µL of water, subjected to a freeze-thaw cycle (-80°C) and centrifuged at 12 800*g* for 10 min. Aliquots (90 µL) of the supernatant were assayed for PNMT activity as described by Pollard et al. (1979). The assay is based on catalysis of the methylation of substrate (phenylethanolamine, 1.8 mM) by [6-methyl-$^3$H]-S adenosyl methionine (10 µM; specific activity=14.3 Ci/mmol) and extraction of tritium-labelled methyl product into toluene/isoamyl alcohol, counted at 50% efficiency. Protein was measured by the method of Lowry et al. (1951). In control cultures containing ethanol in a concentration equal to the highest used to dissolve steroid, PNMT activity was unaffected. Protein levels were unaffected by drug treatments. PNMT activity in control adrenal medullary cells typically ranged from 15-35 pmol methylated product/30 min/µg protein.

### Statistical Analyses

In most cases, [$^3$H]-DEX binding and PNMT data were expressed as a percent of control untreated cultures, and statistical differences were tested using non-parametric

statistics (Kruskall Wallis and Mann-Whitney two sample test, $p<0.05$). Medium cortisol concentration data and [$^3$H]-DEX binding expressed as raw data were analysed using Student's t-test ($p<0.05$), or one way analysis of variance with post hoc Newman-Keul's comparisons ($p<0.05$) where appropriate.

## Results

### Regulation of soluble [$^3$H]-DEX binding by 8 Br-cAMP and -cGMP

To examine whether regulation of soluble GC receptor levels by cAMP occurs in the adrenal medulla, cultured bovine adrenal medullary cells were exposed to the cAMP analogue, 8 Br-cAMP, ($10^{-8}$ to $10^{-3}$ M) for 4 days and [$^3$H]-DEX binding measured. Previous studies in our laboratory have indicated that [$^3$H]-DEX binds with high affinity to the GC receptor in cultured adrenal medullary cells (Betito et al., 1992). In the current study, single point measurements of soluble [$^3$H]-DEX binding were carried out using a concentration ($10^{-8}$ M) of radioligand determined to be saturating in our previous work. Significant loss of soluble [$^3$H]-DEX binding was found following 4 days of treatment with $10^{-4}$ M ($p<0.05$) or $10^{-3}$ M ($p<0.0001$) 8 Br-cAMP, with losses of 32% and 55% respectively (Fig III-1A). Drug treatment did not affect protein levels (data not shown) and there was no correlation between levels of basal [$^3$H]-DEX binding in control cultures and the % decrease in binding following 4 days of treatment with $10^{-3}$ M 8 Br-cAMP.

It has been proposed that the ratio of cAMP to cGMP may be one of the many factors responsible for regulating the levels of GC receptors in a given cell due to opposing effects of the two cyclic nucleotides on GC receptor levels (Oikarinen et al., 1984). In order to examine whether cGMP regulates the GC receptor in adrenal medullary cells in culture, cells were exposed to different concentrations ($10^{-9}$ to $10^{-4}$ M) of the analogue, 8 Br-cGMP, for 4 days and [$^3$H]-DEX binding measured. Significant

($p< 0.02$) losses in [$^3$H]-DEX binding of 31% and 34% were found following $10^{-5}$ M and $10^{-4}$ M 8 Br-cGMP exposure respectively (Fig. III-1B), while no losses in protein levels were observed. In addition, Scatchard analyses of [$^3$H]-DEX binding in cells treated with either 8 Br-cAMP ($10^{-3}$ M) or -cGMP ($10^{-4}$ M) showed no changes in apparent affinity (Kd) compared to vehicle-treated cells (data not shown).

We then examined the effect of elevating endogenous cAMP levels on GC receptor levels. Cells were exposed to $10^{-5}$ M forskolin, to activate cellular adenylate cyclase, for 4 days and soluble [$^3$H]-DEX binding and endogenous cAMP levels measured. We observed a forskolin-induced decrease in soluble [$^3$H]-DEX binding (54% decrease; Table III-1) and confirmed that forskolin elevated endogenous cAMP levels (163% increase; Table III-1). Therefore, either addition of a cAMP analogue or elevation of endogenous cAMP results in a reduction in soluble [$^3$H]-DEX binding in adrenal medullary cells.

In order to determine whether 4 days of exposure to 8 Br-cAMP was necessary to see a loss in soluble [$^3$H]-DEX binding, cells were exposed to $10^{-3}$ M 8 Br-cAMP for varying lengths of time from 12h to 4 days. A significant ($p<0.05$) loss of soluble [$^3$H]-DEX binding was observed as early as 1d (42.5 ± 6.2% decrease; n=14), with similar losses observed at 2 and 4d (40.1 ± 9.1 and 46.0 ± 7.6% decrease; n=14-16) following exposure of adrenal medullary cells to 8 Br-cAMP. However, no significant decrease in [$^3$H]-DEX binding was found at 12h. Therefore, exposure of adrenal medullary cells to 8 Br-cAMP for 12-24h is necessary to decrease soluble [$^3$H]-DEX binding.

**The effect of 8 Br-cAMP and -cGMP on cortisol production**

Adrenal medullary cell cultures routinely contain a small percentage of contaminating adrenocortical cells (Unsicker and Müller, 1981; Banerjee et al., 1985) and cAMP is known to regulate steroid production in cortical cells (Hayashi et al., 1979).

Therefore, we considered the possibility that cAMP may indirectly decrease GC receptor levels in adrenomedullary cultures by increasing cortisol in the medium, thereby leading to downregulation of GC receptors by the released cortisol. Table III-2 shows cortisol levels measured in the medium of cells treated for 4 days with 8 Br-cAMP, forskolin or 8 Br-cGMP. There was a significant ($p<0.05$) increase in the concentration of cortisol in the medium of cells treated with 8 Br-cAMP ($10^{-4}$ or $10^{-3}$ M) or with forskolin ($10^{-5}$ M) compared to vehicle-treated control cultures, with cortisol levels measured in the nanomolar range. In the case of 8 Br-cAMP and of forskolin, all treatments shown to decrease soluble [$^{3}$H]-DEX binding were also found to increase cortisol in the medium [$10^{-4}$ and $10^{-3}$ M 8 Br-cAMP, $10^{-5}$ M forskolin (Table III-2); at 2, 3 and 4d exposure to $10^{-3}$ M 8 Br-cAMP (data not shown)]. The concentration of cortisol in the medium from cells treated with 8 Br-cGMP did not increase above control values at any concentration of the analogue (Table III-2). Neither 8 Br-cAMP or 8 Br-cGMP was found to interfere with the radioimmunoassay for cortisol.

**8 Br-cAMP treatment of chromaffin-enriched cell cultures**

In order to examine more closely the possibility that the effect of cAMP on GC receptor binding is due to cAMP-induced production of cortisol, numbers of contaminating cortical cells were reduced by further purification of medullary cells into a chromaffin-enriched cell population using a differential plating technique (Waymire et al., 1983). Previous characterization by our laboratory (Betito et al., 199?) showed that these chromaffin-enriched cultures contain higher amounts of catecholamines/μg protein than do standard Percoll-isolated cells; additionally, non-chromaffin cell cultures derived from this technique contain only low levels of catecholamines, thus confirming the effectiveness of the purification procedure. A 4 day exposure of the chromaffin-enriched cell cultures to $10^{-3}$ M 8 Br-cAMP resulted in a significant loss (41%, $p<0.05$) in soluble

[$^3$H]-DEX binding (Table III-3). Absolute levels of cortisol in the control media of these cultures were much lower than in the less pure cultures (approximately 2 nM compared to 20 nM, compare Tables III-2 and III-3). However, 8 Br-cAMP was still effective in increasing cortisol levels (to 273% of values for vehicle-treated controls) in the medium of chromaffin-enriched cultures.

**The effect of cortisol on soluble [$^3$H]-DEX binding**

To test if cortisol, in the concentrations found in the medium following 4d 8 Br-cAMP treatment ($10^{-9}$ to $2x10^{-7}$ M), can produce a loss of soluble [$^3$H]-DEX binding, cultured bovine adrenal medullary cells were incubated with cortisol ($10^{-9}$ to $2x10^{-7}$ M) for 4 days and the cells harvested for GC receptor binding. The medium was assayed for cortisol by radioimmunoassay after the 4d incubation to ensure that concentrations of cortisol were, in fact, similar to those observed after 4d 8 Br-cAMP treatment. Medium cortisol concentrations after 4d were found to be similar to the amount of added cortisol, except in cells treated with $2x10^{-7}$ M, where the medium concentration was reduced by 48% (i.e. to approximately $10^{-7}$ M), a concentration similar to that observed in many of the 8 Br-cAMP treated cell culture preparations. Following 4 day exposure of adrenal medullary cells to concentrations of cortisol up to $2x10^{-7}$ M, no decrease in soluble [$^3$H]-DEX binding was observed (Table III-4). Therefore cortisol, in the concentrations found in the medium following 4d 8 Br-cAMP treatment, does not reduce soluble [$^3$H]-DEX binding.

**Prevention of 8 Br-cAMP-induced decreases in [$^3$H]-DEX binding by blockade of cortisol actions**

In one set of studies, we attempted to use the GC receptor antagonist RU 38486 (Philibert and Moguilewsky, 1983; Philibert et al., 1985) to test whether the ability of 8

Br-cAMP to reduce [$^3$H]-DEX binding might be mediated via released cortisol. In pilot studies to test whether RU 38486 could effectively block long-term cortisol-induced decreases in [$^3$H]-DEX binding, cultures were treated for 4 days with either $10^{-5}$ M RU 38486 alone, $10^{-6}$ M cortisol or $10^{-6}$ M cortisol plus $10^{-5}$ M RU 38486. This was followed by a 2h washout period with no added steroid (a condition which allows for recovery of acutely translocated cortisol-bound receptors from an insoluble DNA-bound pool to the soluble receptor pool; Betito et al., 1992). Under these conditions, $10^{-5}$ M RU 38486 alone produced decreases in [$^3$H]-DEX binding that were comparable in magnitude to those produced by $10^{-6}$ M cortisol (RU 38486: 56.4 ± 10.4% decrease, n=19, $p<0.001$; cortisol: 62.3 ± 7.5%, n=12, $p<0.001$). It should be noted that in these experiments, treatment of adrenal medullary cells, with a concentration of cortisol ($10^{-6}$ M) similar to that seen by the medulla in situ (Kitay, 1961; Peytremann et al., 1973; Guidotti and Costa, 1974; Jones et al., 1977), produced a loss in [$^3$H]-DEX binding, in contrast to experiments with lower concentrations ($10^{-9}$ to $2x10^{-7}$ M) of cortisol (Table III-4). The finding that RU 38486 alone decreased [$^3$H]-DEX binding is not surprising since it has been previously reported that RU 38486 is capable of translocating the GC receptor and allows nuclear binding of the receptor without resulting in a functional consequence (Rajpert et al., 1987; Qi et al., 1990; O'Donnell and Meaney, 1991). We were thus unable to utilize this antagonist to block a possible cortisol-induced decrease in soluble [$^3$H]-DEX binding following 8 Br-cAMP treatment. Unfortunately, no appropriate GC receptor antagonist is available at this time for such an experiment.

Serum albumin is an effective means of binding cortisol and should be expected to block any effects of medium cortisol on [$^3$H]-DEX binding in cells treated with 8 Br-cAMP. Therefore, in the next set of experiments we compared effects of bovine serum albumin (BSA) on the loss in [$^3$H]-DEX binding produced by 8 Br-cAMP and by cortisol. For this, cells were treated for 4d with either 8 Br-cAMP ($10^{-3}$ M) or cortisol

($10^{-6}$ M), in the presence or absence of BSA (5 mg/mL), the cells scraped and soluble [$^3$H]-DEX binding measured. Table III-5 shows that the presence of BSA prevents the cortisol-induced loss in soluble [$^3$H]-DEX binding, without affecting the 8 Br-cAMP-induced loss. The two sets of studies in Tables III-4 and III-5 suggest that the cAMP-induced decrease in soluble [$^3$H]-DEX binding is not due to the release of cortisol from contaminating cortical cells.

**Functional consequence of decreased [$^3$H]-DEX binding following 8 Br-cAMP and -cGMP treatment: Loss of induction of PNMT activity**

We have previously shown that a 2h pulse of $10^{-4}$ M cortisol is sufficient to increase the activity of PNMT measured 18h-3 days later in cultured adrenal medullary cells (Betito et al., 1992; 1993). Given the loss in soluble [$^3$H]-DEX binding in response to the cyclic nucleotide second messengers in these cells and that GC biological responses generally depend on the number of GC receptors available (Bourgeois and Newby, 1979; Vanderbilt et al., 1987; Distelhorst, 1989; Dong et al., 1990; Tanaka et al., 1992), we looked at the ability of cells pre-treated with 4 days of 8 Br-cAMP ($10^{-3}$ M) or 8 Br-cGMP ($10^{-4}$ M) to respond to a 2h pulse of $10^{-4}$ M cortisol. PNMT activity was measured 2 days following the 2h pulse of cortisol. Figure III-2 shows that following treatment with either of the cyclic nucleotides, the cells' ability to increase PNMT activity in response to cortisol is significantly lower than that of vehicle-treated control cells. Control levels of PNMT activity did not change following 4d treatment with either the cAMP or cGMP analogue. Therefore, cyclic nucleotide-induced regulation of soluble [$^3$H]-DEX binding translates into reduced GC induction of PNMT activity.

## Discussion

The second messenger, cAMP, has been shown to increase GC receptor and/or mRNA levels in a variety of cells types: rat HTC (Dong et al., 1989; Okret et al., 1991) and hippocampal cells (Mitchell et al., 1992), murine lymphoma cells (Gruol et al., 1986; Gruol et al., 1989), human skin fibroblasts (Oikarinen et al., 1984) and mononuclear leukocytes (Tanaka et al., 1992). Conversely, in the present study using cultured bovine adrenal medullary cells, the cAMP analogue 8 Br-cAMP decreased soluble [$^3$H]-DEX binding by 55% at $10^{-3}$ M of the analogue, with a similar decrease (54%) observed following a forskolin-induced elevation of endogenous cAMP. Scatchard analyses indicated that 8 Br-cAMP ($10^{-3}$ M) as well as 8 Br-cGMP ($10^{-4}$ M), produced no change in the apparent affinity of the GC receptor for [$^3$H]-DEX, suggesting that use of a saturating concentration of the radioligand, as in the majority of these experiments, estimates the density of soluble GC receptors. In only one other study of which we are aware, using the corticotrope tumor cell line AtT20, has exposure of cells to cAMP been previously reported to reduce GC receptor binding (Sheppard et al., 1991). This study reported a decrease in the density of nuclear, cytoplasmic and whole cell [$^3$H]-DEX binding and in GC receptor mRNA levels following elevations in cAMP induced by either CRF or by forskolin. In adrenal medullary cells exposed to 8 Br-cGMP, the analogue caused a decrease in soluble [$^3$H]-DEX binding. On the basis of studies with human skin fibroblasts, where dibutyryl cAMP increases and dibutyryl cGMP decreases whole cell [$^3$H]-DEX binding, it has been suggested that the cellular balance between cAMP and cGMP determines the levels of GC receptor in a given cell type (Oikarinen et al., 1984). However in adrenal medullary cells both cAMP and cGMP analogues decreased soluble [$^3$H]-DEX binding. Therefore, the regulation of GC receptors by cyclic nucleotides appears to be cell type specific.

The mechanism(s) by which cAMP regulates levels of GC receptor binding sites is currently unknown. Conversion of the GC receptor from a non-hormone-binding to the hormone-binding form is known to involve phosphorylation of the GC receptor protein (Singh and Moudgil, 1985; Orti et al., 1989), and has been suggested as a mechanism by which cyclic nucleotides might increase levels of GC binding sites (Gruol et al., 1986). Studies by Dong and coworkers (1989) showing than cAMP can increase the amount of GC receptor protein, as measured by Western immunoblotting, suggest that cAMP may also regulate GC receptor synthesis. However both of these mechanisms provide for increases in GC receptor binding sites, and thus indentical mechanisms cannot account for the decreases in [$^3$H]-DEX binding produced by cAMP in adrenal medullary cells. The long time of exposure (>12h) to 8 Br-cAMP required to produce decreases in [$^3$H]-DEX binding sites in the current study suggest that a mechanism requiring appreciable time, such as regulation of GC receptor synthesis, resulting in decreased receptor levels, could be involved in cAMP regulation of GC receptors in adrenal medullary cells.

The assay for [$^3$H]-DEX binding used in this study measured levels of soluble (cytosolic) non-transformed [$^3$H]-DEX binding sites rather than total binding sites, and does not include the pool of GC receptors that may be transformed and unable to bind ligand. Thus, an alternate mechanism for a cAMP-induced loss in soluble [$^3$H]-DEX binding may be a cyclic nucleotide-induced transformation of GC receptors, or a translocation of GC receptors to an insoluble, DNA-bound pool. However, this mechanism seems unlikely for several reasons. 1) Translocation of GC receptors, at least in response to GCs, is a rapidly occurring event. For example, in our previous studies, incubation of cultured adrenomedullary cells with cortisol for 45 min was sufficient to produce significant loss of soluble [$^3$H]-DEX binding, with levels of these sites returning to control values by 30 min following cortisol removal (Betito et al., 1992). Thus the

long time course of exposure to 8 Br-cAMP required to decrease soluble [$^3$H]-DEX binding, suggests that the analogue is not simply translocating receptors to an insoluble pool. 2) At least in rat hepatocytes, protein kinase C and not cyclic nucleotide dependent protein kinases appear to be involved in the nuclear translocation of GC receptor complexes (Kido et al., 1987). In those cells, nuclear [$^3$H]-DEX binding and DEX-induced tyrosine aminotransferase activity were blocked by the protein kinase C inhibitor, H-7, but not by the cyclic nucleotide-dependent protein kinase inhibitor, H-8. 3) In studies with AtT20 cells, Sheppard et al. (1991) reported that cAMP elevation reduces not only cytoplasmic and whole cell binding of [$^3$H]-DEX, but also reduces nuclear binding of [$^3$H]-DEX, and GC receptor mRNA. 4) Our observation that following cAMP treatment, cortisol was less effective in inducing PNMT activity indicates that cAMP does not increase the proportion of DNA-bound GC receptors able to produce a functional response. However, we cannot eliminate the possibility that cAMP may promote nuclear binding of GC receptors that are unable to induce PNMT. This would be similar to the case reported for human lymphoblastoid cells where RU 38486 is capable of translocating the GC receptor, and allowing binding of the receptor to DNA, without resulting in a functional consequence (Rajpert et al., 1987).

In adrenal medullary cell cultures, dibutyryl cAMP has been shown to maintain contaminating adrenal cortical cells in a differentiated state, while the absence of cAMP results in dedifferentiation of cortical cells into fibroblast-like cells (Unsicker and Ziegler, 1982). In situ, adrenocortical cells respond to cAMP elevation (via ACTH and other modulators) by stimulating steroidogenesis (Grahame-Smith et al., 1967; Hayashi et al., 1979). Therefore, since GCs can regulate their own receptors, we considered the possibility that 8 Br-cAMP might decrease [$^3$H]-DEX binding indirectly via an increase in the levels of cortisol in the medium. We found, in fact, that wherever 8 Br-cAMP reduced soluble [$^3$H]-DEX binding, there was an increase in medium cortisol.

Nonetheless, it is unlikely that the effect of 8 Br-cAMP on soluble [$^3$H]-DEX binding is mediated via cortisol release for the following reasons. 1) Cultures enriched in chromaffin cells by differential plating (Waymire et al., 1983) and containing very low levels of cortisol in the medium, showed similar decreases in soluble [$^3$H]-DEX binding in response to 8 Br-cAMP as did standard cell cultures. 2) Exposure of medullary cells to cortisol for 4d, in a range of concentrations ($10^{-9}$ to $2x10^{-7}$ M) similar to those induced by 8 Br-cAMP, did not reduce [$^3$H]-DEX binding, whereas a reduction was found in response to a higher concentration of cortisol ($10^{-6}$ M), normally seen by the adrenal medulla (Kitay, 1961; Peytremann et al., 1973; Guidotti and Costa, 1974; Jones et al., 1977). 3) Addition of BSA, to adsorb medium cortisol, effectively reversed the loss in [$^3$H]-DEX binding induced by added cortisol, but had no effect on the 8 Br-cAMP-induced loss in [$^3$H]-DEX binding. Therefore, the cAMP-induced loss in soluble [$^3$H]-DEX binding does not appear to be an indirect result of increasing cortisol production from contaminating cortical cells in the adrenal medullary cell cultures. Additionally, effects of 8 Br-cGMP on [$^3$H]-DEX binding and on PNMT activity are also not due to released cortisol, since 8 Br-cGMP did not cause an increase in medium cortisol. However, our finding that 8 Br-cAMP increases cortisol levels in both standard and highly purified adrenal medullary cultures suggests caution in the interpretation of studies using cAMP analogues or forskolin treatment in adrenal medullary cell preparations. It may be important to investigate whether reported effects of cAMP on parameters such as peptide levels or CA synthetic enzymes in medullary cultures are due to release of cortisol, since cortisol is also known to regulate these same parameters.

In terms of the comparison of effects of cAMP and of GCs on GC receptor regulation, our results show that exposure to cAMP on a long term (>12h) basis decreases soluble [$^3$H]-DEX binding sites, while we and others have shown that long-term exposure to GCs also decreases or downregulates GC receptors in adrenal

medullary cells and many other cell types (Svec and Rudis, 1981; Sapolsky et al., 1984; McIntyre and Samuels, 1985; Svec, 1985a; Svec, 1985b; Miller et al., 1990; O'Donnell and Meaney, 1991; present study). Thus long-term effects of cAMP and of the GCs on soluble GC receptor levels appear to be similar, consistent with observations that cAMP and GCs appear to have similar effects on many parameters in the adrenal medulla. However, in terms of functional consequences to the adrenal chromaffin cell, the pattern of exposure to cAMP and the GCs appears to play a crucial role in determining the final functional consequence to the cell. When medullary cells were first exposed to 8 Br-cAMP for 4 days to decrease GC receptors, followed by a 2h exposure to cortisol, effects of 8 Br-cAMP on PNMT induction were opposed to those of cortisol. Cells pre-treated with 8 Br-cAMP induced PNMT in response to cortisol less effectively than did vehicle pre-treated cells. This finding supports the demonstration that in tissues other than the adrenal medulla, the magnitude of the biological response to GCs is proportional to the levels of GCs in a given cell (Bourgeois and Newby, 1979; Vanderbilt et al., 1987; Distelhorst, 1989; Dong et al., 1990; Tanaka et al., 1992). Previously, it had not been possible to examine whether dynamic changes in GC receptor levels could alter GC receptor-mediated function in adrenal medullary cells, since the prevailing notion from both in vitro and in vivo studies had been that long-term (18h-2d) exposure of medullary cells to GCs was necessary to elevate PNMT activity and/or catecholamine levels (Hersey and DiStefano, 1979; Kelner and Pollard, 1985; Nawata et al., 1985). Thus, it was unknown if drug-induced GC receptor losses would remain as such over the 2d period of GC exposure thought to be needed for measurements of GC-mediated function. This technical problem has been solved by our recent in vitro evidence showing that a short (15 min-2h) pulse of GCs can in fact elevate adrenal medullary PNMT (measured 2-3d later; Betito et al., 1992; 1993). This has provided us with the experimental opportunity to show that dynamic changes in GC receptor levels (produced by cAMP and cGMP) can

alter GC receptor mediated function in medullary cells. Therefore, our results provide evidence for a much more dynamic regulation of adrenal medullary cathecholamine synthesis, by GCs and other modulators such as cyclic nucleotides, than was previously thought, determined by the timing and pattern of exposure to these agents.

In summary, the present study shows that both cyclic nucleotide second messengers cAMP and cGMP decrease soluble [$^3$H]-DEX binding in cultured bovine adrenal medullary cells. We have observed a cAMP-induced production of cortisol in the medium of treated adrenal medullary cells, although this production of steroid does not appear to be responsible for the loss in soluble [$^3$H]-DEX binding. However, this does raise caution in the interpretation of studies using cAMP analogues or forskolin treatment in bovine adrenal medullary cell preparations. Finally, the loss in soluble [$^3$H]-DEX binding in response to elevation of intracellular cAMP or cGMP translates into a reduction in the functional consequence of GC receptor binding in adrenomedullary cells, the loss in the ability to fully induce the activity of PNMT in response to a GC pulse following 4d exposure to 8 Br-cAMP or -cGMP. This is the first report to show a relationship between the levels of GC receptor and the inducibility of the GC-regulated enzyme, PNMT, in adrenal medullary cells in culture. Our results indicate that the pattern and timing of exposure of adrenomedullary cells to cAMP and GCs may be important determinants of the cellular response to these agents.

**Figure III-1. 4 day treatment of adrenal medullary cells with 8 bromo-cAMP (8 Br-cAMP) or 8 bromo-cGMP (8 Br-cGMP) reduces [$^3$H]-DEX binding.** A) Adrenal medullary cells were treated with various concentrations ($10^{-8}$ to $10^{-3}$ M) of 8 Br-cAMP for 4 days and soluble [$^3$H]-DEX binding measured. Each point represents the mean (± SEM) value for 12-18 plates from 3 preparations for all except $10^{-3}$ M, which is the mean (± SEM) for 44 plates from 7 preparations (*$p<0.03$, **$p<0.0001$ versus control untreated cultures). B) Cells were treated with various concentrations ($10^{-9}$ to $10^{-4}$ M) of 8 Br-cGMP for 4 days and [$^3$H]-DEX binding measured. Each bar represents the mean (± SEM) from 12-26 plates from 3-5 cell preparations (**$p<0.02$ versus control untreated cultures).

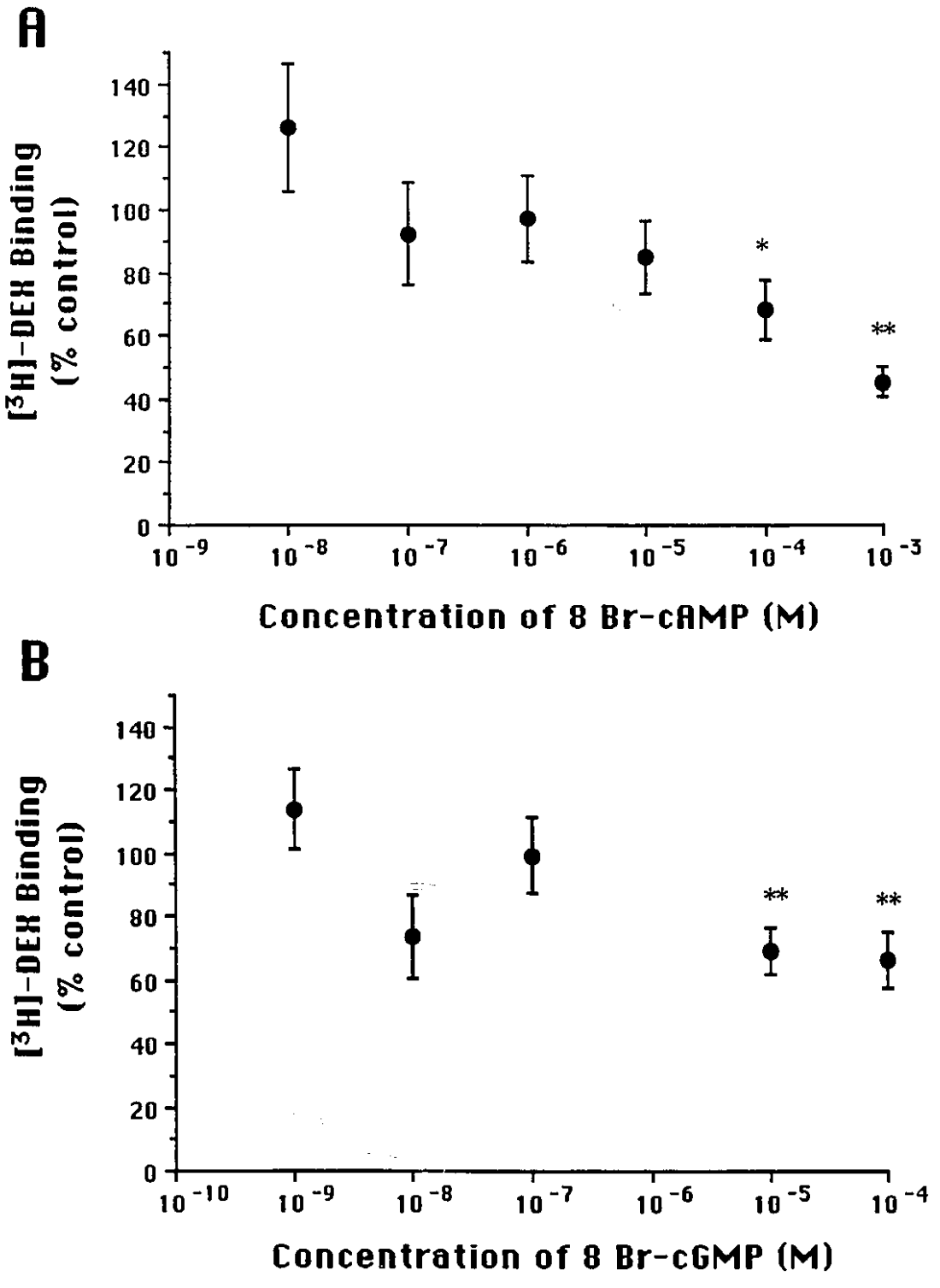

A
[3H]-DEX Binding
(% control)
140
120
100
80
60
40
20
0
*
**
10 -9
10 -8
10 -7
10 -6
10 -5
10 -4
10 -3
Concentration of 8 Br-cAMP (M)
B
[3H]-DEX Binding
(% control)
140
120
100
80
60
40
20
0
**
**
10 -10
10 -9
10 -8
10 -7
10 -6
10 -5
10 -4
Concentration of 8 Br-cGMP (M)

**Figure III-2. Cyclic nucleotide treated cells have a reduced ability to respond to a 2h pulse of cortisol by increasing PNMT activity.** Adrenal medullary cells were pretreated with either serum free medium ("control"), 8 Br-cAMP ($10^{-3}$ M), or 8 Br-cGMP ($10^{-4}$ M) for 4 days. The medium was removed from all culture plates, and the plates were pulsed with either serum free control medium ("2h vehicle pulse" for each condition; open bars) or serum free medium containing $10^{-4}$ M cortisol for 2 hours [2h cortisol ($10^{-4}$ M) pulse; closed bars]. All plates received serum free medium for 2 days, the cells were then scraped and PNMT activity measured. Results for all groups are expressed as a % of activity measured in control untreated cells (i.e. 4 day treatment with medium followed by 2h vehicle pulse, control open bar). Four day treatment with either 8 Br-cAMP or 8 Br-cGMP had no significant effect on control PNMT activity (compare open bars). Thus expressing data for 8 Br-cAMP/2h cortisol treated cells either as a % of values for control untreated cells, as in Fig. 4, or as a % of values for 8 Br-cAMP/2h vehicle treated cells, yielded the same finding, i.e., 4 day pre-treatment with 8 Br-cAMP results in a reduced ability for cortisol to induce PNMT, in comparison to cells pre-treated for 4 days with medium. A similar argument holds for 8 Br-cGMP. Each bar represents the mean (± SEM) value for the following numbers of plates: 53-61 for control condition, 25-32 for 8 Br-cAMP condition, 23 for 8 Br-cGMP condition from 3 cell preparations. [† $p<0.05$ compared to control condition, 2h pulse (closed bar), **$p<.002$ compared to treatment condition, no pulse (open bar)].

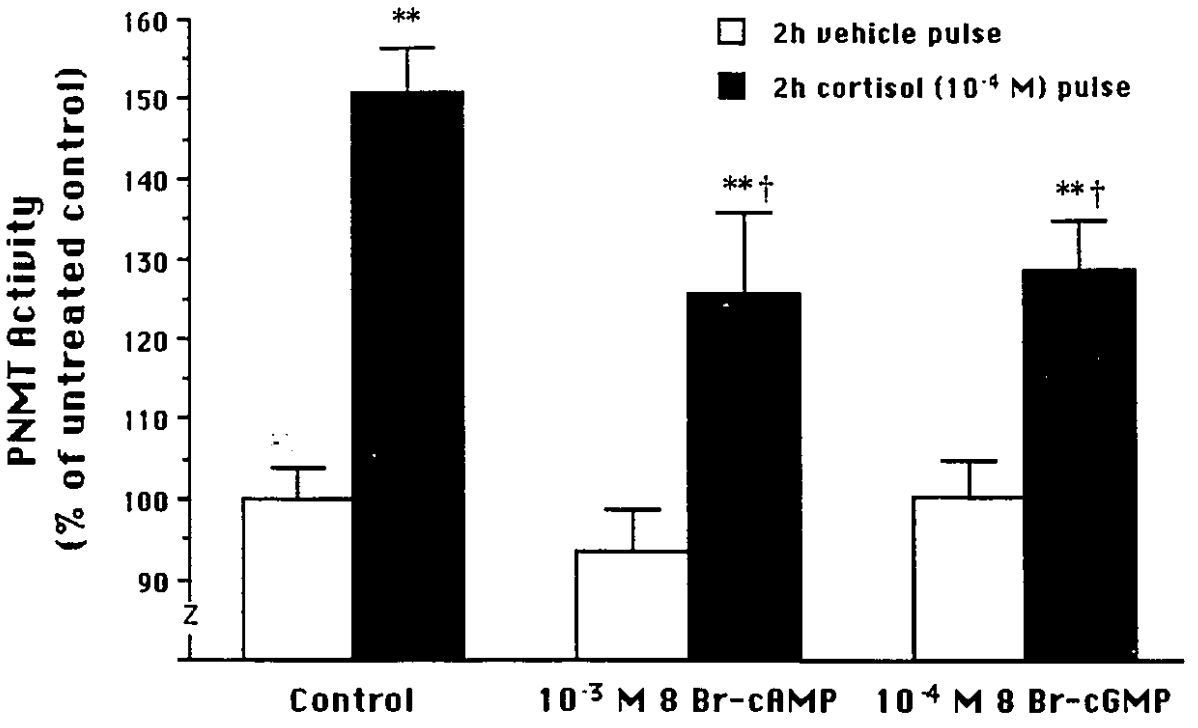

PNMT Activity
(% of untreated control)
160
150
140
130
120
110
100
90
**
**†
**†
2h vehicle pulse
2h cortisol (10⁻⁴ M) pulse
Control
10⁻³ M 8 Br-cAMP
10⁻⁴ M 8 Br-cGMP

**Table III-1. Elevation of cAMP levels by forskolin decreases [$^3$H]-DEX binding.**

| | [$^3$H]-DEX binding (% control) | cAMP Levels (% control) |
|---|---|---|
| Control | 100.0 ± 10.9 (15) | 100.0 ± 5.8 (14) |
| Forskolin ($10^{-5}$ M) | 45.9 ± 12.3 (12)** | 262.5 ± 17.6 (14)** |

Adrenal medullary cells were treated with $10^{-5}$ M forskolin for 4 days, and [$^3$H]-DEX binding or cAMP levels were measured. Data are expressed as the mean (± SEM) value for (n) plates from 2 preparations (** $p<0.005$ versus control untreated cultures).

**Table III-2. Cortisol concentration in the medium of cells treated for 4 days with forskolin, 8 Br-cAMP or 8 Br-cGMP.**

| | Cortisol Concentration ( x $10^{-9}$ M ) |
|---|---|
| **8 Br-cAMP:** | |
| Control | 11.2 ± 1.2 (4) |
| 8 Br-cAMP ($10^{-8}$ M) | 8.1 ± 0.7 (4) |
| 8 Br-cAMP ($10^{-7}$ M) | 13.1 ± 1.1 (4) |
| 8 Br-cAMP ($10^{-6}$ M) | 12.8 ± 0.7 (4) |
| 8 Br-cAMP ($10^{-5}$ M) | 17.9 ± 2.8 (4) |
| 8 Br-cAMP ($10^{-4}$ M) | 45.7 ± 4.4 (4)* |
| Control | 28.8 ± 3.5 (20) |
| 8 Br-cAMP ($10^{-3}$ M) | 99.5 ± 10.3 (20)** |
| **Forskolin:** | |
| Control | 47.3 ± 8.3 (5) |
| Forskolin ($10^{-5}$ M) | 187.5 ± 5.7 (5)** |
| **8 Br-cGMP:** | |
| Control | 10.1 ± 1.6 (4) |
| 8 Br-cGMP ($10^{-9}$ M) | 10.2 ± 1.2 (4) |
| 8 Br-cGMP ($10^{-8}$ M) | 7.5 ± 0.7 (4) |
| 8 Br-cGMP ($10^{-7}$ M) | 6.5 ± 0.3 (4) |
| 8 Br-cGMP ($10^{-4}$ M) | 10.5 ± 0.4 (4) |
| Control | 17.3 ± 1.1 (26) |
| 8 Br-cGMP ($10^{-5}$ M) | 17.2 ± 1.1 (26) |

Adrenal medullary cells were treated with either 8 Br-cAMP ($10^{-8}$ to $10^{-3}$ M), forskolin ($10^{-5}$ M), or 8 Br-cGMP ($10^{-9}$ to $10^{-4}$ M) for 4 days, the medium removed and assayed for cortisol using a radioimmunoassay. Data are expressed as the mean ± S.E.M. for (n) plates from either 1 preparation for the forskolin, 8 Br-cAMP ($10^{-8}$ to $10^{-4}$ M), or 8 Br-cGMP ($10^{-9}$ to $10^{-7}$ M and $10^{-4}$ M) treatments or 3 preparations for the 8 Br-cAMP ($10^{-3}$ M) and 8 Br-cGMP ($10^{-5}$ M) treatments (**p=0.0001, *p<0.05 vs control).

**Table III-3. [$^3$H]-DEX binding and medium cortisol in chromaffin-enriched cell cultures treated for 4 days with $10^{-3}$ M 8 Br-cAMP.**

| | [$^3$H]-DEX binding (% control) | Cortisol Concentration (x $10^{-9}$ M) |
|---|---|---|
| Control | 100.0 ± 13.7 (18) | 1.96 ± 0.37 (14) |
| 8 Br-cAMP ($10^{-3}$ M) | 59.3 ± 11.3 (16)* | 7.31 ± 1.24 (14)** |

Percoll-isolated adrenal medullary cells were further purified into a chromaffin-enriched cell population by differential plating as described in Experimental Procedures, and treated with 8 Br-cAMP ($10^{-3}$ M) for 4 days. The medium was removed and assayed for cortisol concentration, and the cells were scraped and [$^3$H]-DEX binding measured. Data are expressed as the mean ± S.E.M. for (n) plates from 2 preparations (**$p<0.001$, *$p<0.05$ vs control).

**Table III-4. [$^3$H]-DEX binding in cells treated for 4 days with various concentrations of cortisol.**

| | | [$^3$H]-DEX binding (% control) |
|---|---|---|
| Added Cortisol | $10^{-9}$ M | 92.6 ± 13.3 (15) |
| | $10^{-8}$ M | 117.5 ± 22.4 (16) |
| | $5x10^{-8}$ M | 112.8 ± 14.8 (20) |
| | $2x10^{-7}$ M | 112.6 ± 25.9 (12) |

Adrenal medullary cells were treated with cortisol ($10^{-9}$ to $2x10^{-7}$ M) for 4 days, the cells scraped and [$^3$H]-DEX binding measured. Data are expressed as the mean ± SEM for (n) plates from 2 preparations. No significant differences between groups were found.

**Table III-5. The presence of bovine serum albumin in the medium of cells treated with 8 Br-cAMP does not prevent the loss in soluble [$^3$H]-DEX binding.**

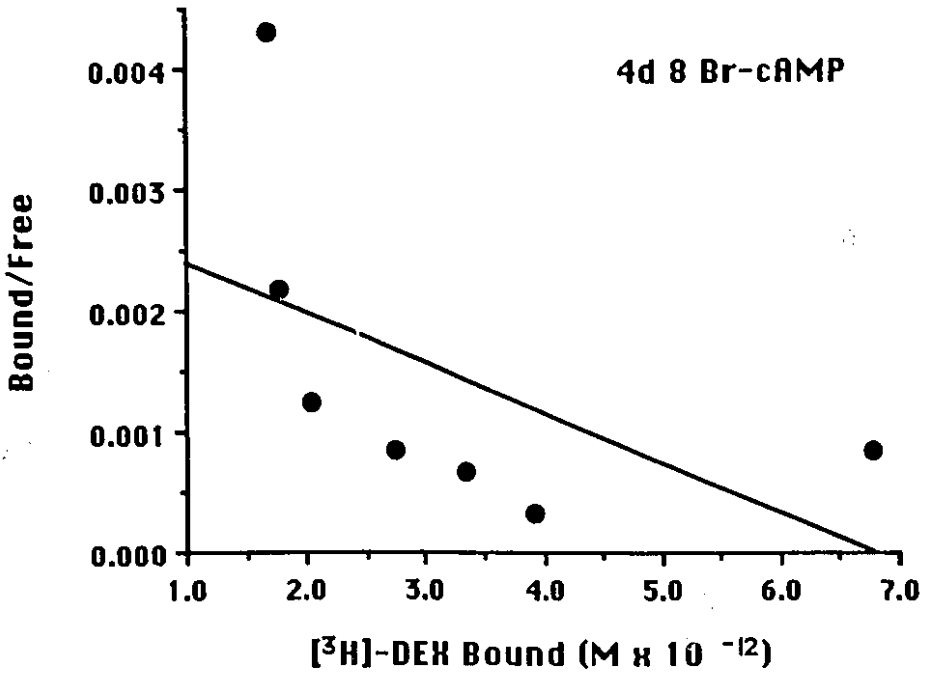
| | [$^3$H]-DEX binding (fmol/$10^6$ cells) |
|---|---|
| Control | 1.057 ± 0.153 (27) |
| 8 Br-cAMP ($10^{-3}$ M) | 0.604 ± 0.118 (15)* |
| 8 Br-cAMP ($10^{-3}$ M) + BSA | 0.663 ± 0.102 (26)* |
| Cortisol ($10^{-6}$ M) | 0.502 ± 0.075 (25)* |
| Cortisol ($10^{-6}$ M) + BSA | 1.053 ± 0.092 (31)† |

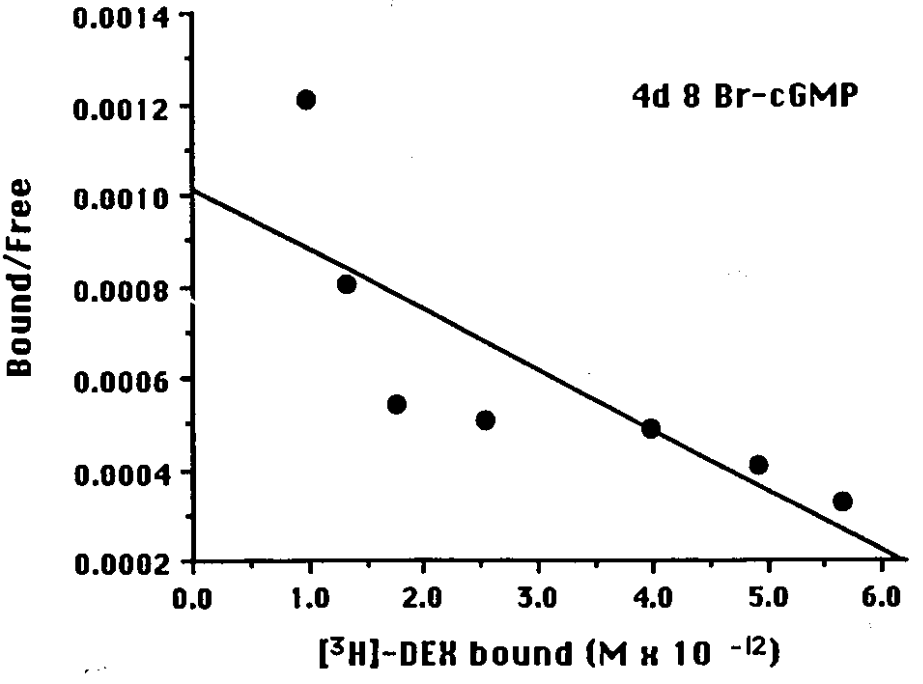
Cells were treated with $10^{-3}$ M Br-cAMP, or $10^{-6}$ M cortisol, for 4 days in the presence or absence of 5 mg/mL BSA, and soluble [$^3$H]-DEX binding measured. Data are expressed as the mean (± SEM) value for (n) plates, from 3-4 cell preparations (*$p<0.05$ versus control untreated cultures, † $p<0.05$ versus cortisol alone).

## Appendix A. Additional Data for Chapter III.

The following figure contains data mentioned in the text as unpublished data (see page III-11), and is not included in the published manuscript.

**Figure III-3. 4 day treatment of adrenal medullary cells with 8 bromo-cAMP (8 Br-cAMP) or 8 bromo-cGMP (8 Br-cGMP) does not affect Kd for [$^3$H]-DEX binding.** Adrenal medullary cells were treated with 8 Br-cAMP ($10^{-3}$ M), or 8 Br-cGMP ($10^{-4}$ M) for 4 days. Saturation analyses of [$^3$H]-DEX binding to cytosolic fractions of treated cells were obtained. Scatchard transformations of the data were performed on 3 samples for each drug and for vehicle-treated cells, and the apparent affinity constants (Kd) calculated. No significant differences between the Kds from vehicle-, 8 Br-cAMP- and 8 Br-cGMP-treated cells was found.




| | |
|---|---|
| Control Average Kd | 3.48±0.85 |
| 8 Br-cAMP Average Kd | 3.06±0.17 |
| 8 Br-cGMP Average Kd | 5.36±2.25 |

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# Chapter IV. Brief Cortisol Exposure Elevates Adrenal Phenylethanolamine N-Methyltransferase After a Necessary Lag Period

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**Abstract**

The present study, using bovine adrenal medullary cells, characterized in detail the time course of regulation of phenylethanolamine N-methyltransferase activity following brief glucocorticoid exposure. Cortisol pulses ($10^{-4}$ M and $10^{-5}$ M), as short as 15 min, increased phenylethanolamine N-methyltransferase activity measured 2 days following cortisol exposure, with a required lag period of 18 h or more. Phenylethanolamine N-methyltransferase activity was increased 2 days following brief (2 h) exposure to cortisol in concentrations that reach the medulla in vivo ($10^{-6}$ M to $10^{-4}$ M). Phenylethanolamine N-methyltransferase activity following both continuous and 2 h pulses of $10^{-5}$ M cortisol were reduced by the glucocorticoid receptor antagonist, RU 38486. A 2 h pulse of nicotine ($10^{-5}$ M) increased phenylethanolamine N-methyltransferase activity with a lag period of at least 18 h, while combination treatment of nicotine and cortisol ($10^{-4}$ M) produced significantly higher increases in phenylethanolamine N-methyltransferase compared to either treatment alone. Therefore, this study provides novel in vitro evidence for the regulation of adrenomedullary phenylethanolamine N-methyltransferase activity, following a necessary lag period, by acute changes in both cortisol and nicotine.

**Key Words:** glucocorticoid receptors; adrenal medulla; phenylethanolamine N-methyltransferase; nicotine; catecholamines.

## Introduction

Both glucocorticoids and catecholamines, two major hormones involved in the body's response to stress, originate from the adrenal gland. Due to the anatomical association between the adrenal medulla and the adrenal cortex, the medulla is influenced by the high levels of glucocorticoids secreted by the cortex into the intra-adrenal portal system perfusing the medulla. Glucocorticoids have been shown to regulate the activity and/or mRNA of phenylethanolamine N-methyltransferase (E.C. 2.1.1.28), the final enzyme in the catecholamine biosynthetic pathway converting noradrenaline to adrenaline, both in vivo (Wurtman and Axelrod 1965, 1966; Pohorecky and Wurtman, 1971; Ciaranello and Black, 1971; Stachowiak et al., 1988) and in vitro (Hersey and DiStefano, 1979; Kelner and Pollard, 1985; Wan and Livett, 1989; Stachowiak et al., 1990a; Ross et al., 1990; Betito et al., 1992).

In terms of the time course and proposed physiological role of glucocorticoids in the regulation of phenylethanolamine N-methyltransferase activity, glucocorticoids have long been thought to play a role in the long-term maintenance of steady state phenylethanolamine N-methyltransferase levels (Wurtman and Axelrod, 1966; Fuller and Hunt, 1967; Thoenen et al., 1970; Ciaranello and Black, 1971); this is based on the observation that hypophysectomy, with the resultant decrease in adrenocorticotropic hormone and glucocorticoid secretion, reduced adrenomedullary phenylethanolamine N-methyltransferase activity (Wurtman and Axelrod, 1965, 1966). Subsequent studies also provided evidence that prolonged (days to weeks) elevation in glucocorticoid secretion may play a role in increasing phenylethanolamine N-methyltransferase activity on a long-term basis. Thus, measures which produced long-term increases in circulating glucocorticoid levels in the intact animal, such as prolonged adrenocorticotropic hormone administration (Vernikos-Danellis et al., 1968; Simonyi et al., 1985), chronic stress (Kvetnansky et al., 1970), high salt intake in hypertensive rats (Saavedra et al., 1983), or mother-infant separation (Breese et al., 1973), were shown to increase

phenylethanolamine N-methyltransferase activity. Similarly, in vitro experiments using bovine adrenal medullary cells in culture showed that 18 h to 2 days of continuous exposure to glucocorticoids produced increases in phenylethanolamine N-methyltransferase activity or catecholamine levels, with shorter times of exposure being ineffective (Hersey and DiStefano, 1979; Kelner and Pollard, 1985; Nawata et al., 1985). Since the duration of increased glucocorticoid levels during an acute stressor is generally much briefer (<2 h) than this, glucocorticoids had previously been thought to play little role in the regulation of phenylethanolamine N-methyltransferase activity following more acute stress. In contrast to this view, a recent study in our laboratory showed that a 2 h pulse of cortisol could increase phenylethanolamine N-methyltransferase activity in cultured adrenal medullary cells, if the activity was measured 3 days later (Betito et al., 1992). This suggested that glucocorticoids could play a more prominent role in dynamic changes in phenylethanolamine N-methyltransferase activity following an acute stressor, than had previously been thought. One of the aims of the present study was to provide a detailed characterization of the time constraints imposed on regulation of phenylethanolamine N-methyltransferase activity following brief glucocorticoid exposure. For this, we examined both the time of cortisol exposure and the lag period following cortisol exposure, that are required to observe increases in phenylethanolamine N-methyltransferase activity in cultured bovine adrenal medullary cells. We show that a cortisol pulse as short as 15 minutes is sufficient to elevate phenylethanolamine N-methyltransferase activity 2 days later, with a short pulse requiring a waiting period of at least 18 h following exposure to elevate adrenal phenylethanolamine N-methyltransferase.

In an acute stress situation, there is short-term exposure of the adrenal medulla to splanchnic nerve contents as well as to glucocorticoids. Secretion of catecholamines from the adrenal medulla is mostly in response to the release of splanchnic nerve acetylcholine, acting at nicotinic receptors (Douglas and Rubin, 1961; Douglas, 1966). Although the regulation of adrenomedullary phenylethanolamine N-methyltransferase activity is

predominantly by glucocorticoids acting at glucocorticoid receptors, continuous exposure to cholinergic nicotinic agonists [18-24 h (Evinger et al., 1988) or 6-18 h (Stachowiak et al., 1990a)] has also been shown to increase phenylethanolamine N-methyltransferase mRNA in bovine adrenal medullary cells. In the present study, we provide the first evidence that a 2 h pulse of nicotine is sufficient to increase phenylethanolamine N-methyltransferase activity 18 to 48 h following the beginning of exposure to the pulse, and that there is a further nicotine-induced increase in phenylethanolamine N-methyltransferase activity in the presence of cortisol. Therefore, using an in vitro model of the adrenal medulla, we demonstrate a long-term regulation of phenylethanolamine N-methyltransferase activity by short-term exposure to either cortisol or a nicotinic agonist, both of which are released within a similar brief time frame following an acute stressor in the in vivo situation.

## Materials and Methods

### Cell Culture

Adrenal medullary cells were isolated by retrograde perfusion of bovine adrenal glands with collagenase and DNase I, and purification of isolated cells on Percoll density gradients as described by Livett (1984). Medullary cell cultures prepared by the above protocol contain approximately 85-90% chromaffin cells and 10-15% non-chromaffin cells (cortical and endothelial cells, fibroblasts; Banerjee et al., 1985; Unsicker and Muller, 1981). The cells were plated on collagen-coated 24 well plates ($2.5 \times 10^5$ cells/well) or 35 $mm^2$ culture plates ($10^6$ cells/plate) and maintained at 37°C in 5% $CO_2$ in air in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Burlington, Ontario) supplemented with 10% fetal calf serum, 1% glucose, 100 µg/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml gentamycin sulfate, 2.5 µg/ml fungizone and $10^{-5}$ M cytarabine (Cytosar, Sigma, St. Louis, MO; used to prevent growth of rapidly dividing non-

chromaffin cells). Using this protocol, cells are isolated from several glands and pooled. Therefore, a "preparation" of adrenal chromaffin cells refers to all of the plates generated, on a single day, by the complete isolation and purification procedure. For all studies, the cells were used between day 6-10 of culture and were maintained in control medium [50% DMEM, 50% Ham's F12 Nutrient Mixture (GIBCO), supplemented with 10% fetal calf serum with antibiotics and Cytosar]. Steroids were dissolved in ethanol, and nicotine dissolved in water, and appropriate vehicle controls performed with all experiments.

**Phenylethanolamine N-methyltransferase activity**

Adrenal medullary cells, maintained in control medium, were treated with cortisol (Aldrich, Milwaukee, WI), nicotine (Sigma, St. Louis, MO), or the glucocorticoid receptor antagonist 17ß-hydroxy-11ß,-4-dimethylaminophenyl-17a -propynyl estra-4,9-diene-3-one (RU 38486; Philibert and Moguilewsky, 1983; Philibert et al., 1985) (generously provided by D. Philibert, Roussel-Uclaf, Romainville, France), for the amount of time indicated in the figure legends. Following this, cells were washed twice with control medium and allowed to incubate in drug-free medium for the required lag period. Cells were then scraped into water (150 µl/well for 24 well dishes; 500 µl for 35 $mm^2$ dishes), subjected to a freeze-thaw cycle (-80°C) and centrifuged at 12 800*g* for 10 min. Aliquots (90 µl) of the supernatant were assayed for phenylethanolamine N-methyltransferase activity as described by Pollard et al. (1979). The assay is based on catalysis of the methylation of substrate (phenylethanolamine, 1.8 mM) by [6-methyl-$^3$H]S adenosyl methionine (10 µM; specific activity=14.3 Ci/mmol) and extraction of tritium labelled methyl product into toluene/isoamyl alcohol, counted at 50% efficiency. Protein was measured by the method of Lowry et al. (1951). In control cultures containing ethanol in a concentration equal to the highest used to dissolve steroid, phenylethanolamine N-methyltransferase activity was unaffected. Phenylethanolamine N-methyltransferase activity is expressed as a percentage of that in control untreated

cultures, with control cultures typically containing 15-50 pmol/30 min/μg protein of phenylethanolamine N-methyltransferase activity.

### Statistical Analyses

Raw data were expressed as percentage of control untreated cultures from the same cell preparation, combined across cell culture preparations, and analyzed using non-parametric statistics (Mann-Whitney two sample test, $P<0.05$, and Kruskall-Wallis multiple sample test, $P<0.05$).

## Results

### Phenylethanolamine N-methyltransferase activity 2 days following a cortisol pulse

Continuous exposure of adrenal medullary cells to glucocorticoids for 18-24 h has previously been shown to increase phenylethanolamine N-methyltransferase activity (Hersey and DiStefano, 1979; Kelner and Pollard, 1985). In situations such as acute stress, exposure to glucocorticoids is of shorter duration. We have recently measured an increase in phenylethanolamine N-methyltransferase activity after a steroid-free lag period of several days in adrenal medullary cells exposed to a short (2 h) pulse of glucocorticoids (Betito et al., 1992). The initial experiments in this study examined how brief the exposure to glucocorticoids can be, in order for one to still observe an increase in phenylethanolamine N-methyltransferase activity 2 days later. For this, at time 0, cells were exposed to $10^{-5}$ M or $10^{-4}$ M cortisol, or control medium for various times indicated in fig. IV-1. Following glucocorticoid exposure, the medium was replaced with control medium and phenylethanolamine N-methyltransferase activity measured at time 0 + 2 days. Separate cell cultures were also exposed to a pulse of vehicle (ethanol), with no resulting change in the activity of the enzyme found under any condition (this data is not

included in the graph for the sake of simplicity). A pulse of cortisol as short as 15 min was able to elevate phenylethanolamine N-methyltransferase activity measured 2 days later. Exposure to cortisol for time periods ranging from 15 min to 24 h were all effective in increasing phenylethanolamine N-methyltransferase activity measured at time 0 + 2 days.

**Lag period requirement for a pulse of cortisol to increase phenylethanolamine N-methyltransferase activity**

Continuous exposure to glucocorticoids for 18 h to 2 days has previously been shown to be necessary for glucocorticoid regulation of adrenal medullary phenylethanolamine N-methyltransferase activity (Pohorecky and Wurtman, 1971; Hersey and DiStefano, 1979; Kelner and Pollard, 1985). In order to examine whether a similar period of time is required to observe changes in phenylethanolamine N-methyltransferase activity following a short pulse of glucocorticoids, medullary cells were exposed to cortisol ($10^{-4}$ M) for 2 h (fig. IV-2A). The medium was replaced with cortisol-free medium and the cells were assayed for phenylethanolamine N-methyltransferase activity at either 12, 18, 24, or 48 h after the beginning of the 2 h cortisol pulse. Fig. IV-2A shows that a short (2 h) pulse of glucocorticoids significantly ($P<0.01$) increased phenylethanolamine N-methyltransferase activity after a lag period of 18 h or more following the beginning of the cortisol pulse; however, following the shorter lag period of 12 h, there was no measurable increase in phenylethanolamine N-methyltransferase activity. Since shorter times of exposure to a cortisol pulse also results in increases in phenylethanolamine N-methyltransferase activity 2 days later (see fig. IV-1), a shorter pulse (30 min) was also used to examine the requirement for a lag period (fig. IV-2B). As for the 2 h pulse, a 30 min pulse of cortisol required 18 h or more following the beginning of cortisol exposure to increase phenylethanolamine N-methyltransferase activity, with no increase observed at 12 h. Note that in both fig. IV-

2A and IV-2B, the increase in phenylethanolamine N-methyltransferase activity tended to grow larger at each successive time point where phenylethanolamine N-methyltransferase activity was measured.

**Phenylethanolamine N-methyltransferase activity following a pulse of various concentrations of cortisol**

In the previous figures, a short pulse of high concentrations of cortisol was shown to increase phenylethanolamine N-methyltransferase activity 2 days later. This experiment tested the range of cortisol concentrations able to increase phenylethanolamine N-methyltransferase activity following short pulse (2 h) exposure. For this, cells were exposed to different concentrations of cortisol ($10^{-9}$ to $10^{-4}$) for 2 h, the medium replaced, and the cells assayed for phenylethanolamine N-methyltransferase activity 2 days later (fig. IV-3). Cortisol concentrations in the lower range ($10^{-9}$ to $10^{-7}$ M) did not elevate phenylethanolamine N-methyltransferase activity. A pulse of cortisol in the concentration range seen physiologically by the adrenal medulla both under basal and stress conditions ($10^{-6}$ to $10^{-4}$ M; Kitay, 1961; Peytremann et al., 1973; Guidotti and Costa, 1974; Jones et al., 1977) significantly elevated phenylethanolamine N-methyltransferase activity compared to untreated control cells.

**Phenylethanolamine N-methyltransferase activity following a pulse of cortisol in the presence of the glucocorticoid receptor antagonist, RU 38486**

To test whether the increase in phenylethanolamine N-methyltransferase caused by a 2 h pulse of glucocorticoids, as well as by continuous 2 day exposure, was glucocorticoid receptor mediated, medullary cells were treated with $10^{-5}$ M cortisol for 2 h, or continuously for 2 days, in the presence or absence of $10^{-5}$ M of the glucocorticoid receptor antagonist, RU 38486 (Philibert and Moguilewsky, 1983; Philibert et al., 1985).

The medium was replaced and the cells assayed for phenylethanolamine N-methyltransferase activity 2 days following the beginning of treatment in the case of the 2 h pulse, or at the end of the 2 days in the case of continuous exposure (Table IV-1). The increase in phenylethanolamine N-methyltransferase activity caused by continuous 2 day exposure to cortisol was significantly reduced by the glucocorticoid receptor antagonist, RU 38486. In addition, the cortisol-induced increase in phenylethanolamine N-methyltransferase activity following a 2 h pulse of cortisol was significantly blocked by the glucocorticoid receptor antagonist. Phenylethanolamine N-methyltransferase activity was not affected by exposure to RU 38486 alone (data not shown).

**The effect of pretreatment with moderate concentrations of cortisol on the ability of a 2 h pulse of cortisol to increase phenylethanolamine N-methyltransferase activity**

All previous experiments in this study were carried out on cells maintained in a medium containing fetal bovine serum (10%) with no added steroid. Typically, commercial bovine serum contains nanomolar levels of corticosteroids (GIBCO, personal communication; unpublished observations). However, physiologically, the adrenal medulla is exposed to continuous basal levels of glucocorticoids in the order of $10^{-6}$ M or higher (Kitay, 1961; Peytremann et al., 1973; Jones et al., 1977), probably rising to $10^{-4}$ M during stress (Jones et al., 1977). To more closely mimic the physiological situation, this experiment tested whether adrenal medullary cells would still increase phenylethanolamine N-methyltransferase activity in response to a pulse of a high concentration of cortisol ($10^{-4}$ M) following pretreatment with a lower (basal) concentration of cortisol ($10^{-6}$ M). For this, adrenal medullary cells were pretreated with medium containing $10^{-6}$ M cortisol, or with medium containing vehicle (control medium), for 4 days. The cells were then exposed to a 2 h pulse of $10^{-4}$ M cortisol or control medium (vehicle) as in the previous studies, and phenylethanolamine N-methyltransferase

activity measured 2 days later. As expected, continuous cortisol treatment ($10^{-6}$ M) for 4 days significantly increased phenylethanolamine N-methyltransferase activity measured 2 days after removal of the cortisol (fig. IV-4, compare open bars). Cells pretreated with control medium and exposed to a 2 h pulse of $10^{-4}$ M cortisol also showed the expected increase in phenylethanolamine N-methyltransferase activity (compare open bar to filled bar on left side of fig. IV-4). Cortisol ($10^{-6}$ M)-pretreated cells exposed to a 2 h pulse of $10^{-4}$ M cortisol showed a significant ($P<0.001$) increase in phenylethanolamine N-methyltransferase activity (compare open bar to filled bar on right side of fig. IV-4). The increase in phenylethanolamine N-methyltransferase activity in response to the 2 h cortisol pulse was similar for both the control and cortisol pretreatment conditions (30-40% increase; compare filled bar to open bar for each pretreatment condition).

**The effect of a 2 h pulse of nicotine on phenylethanolamine N-methyltransferase activity**

In medullary cell cultures, continuous long-term (6-24 h) exposure to nicotine has been shown to elevate phenylethanolamine N-methyltransferase mRNA (Evinger et al., 1988; Stachowiak et al., 1990a). To test whether more acute nicotinic receptor activation regulates phenylethanolamine N-methyltransferase activity, the ability of short-term nicotine exposure to increase phenylethanolamine N-methyltransferase activity was examined. To test whether nicotine and glucocorticoid effects might be additive, the ability of nicotine to increase phenylethanolamine N-methyltransferase activity in the presence of cortisol was also investigated. Adrenal medullary cells were treated with a 2 h pulse of $10^{-5}$ M nicotine or $10^{-4}$ M cortisol, or a combination of both, the medium removed and phenylethanolamine N-methyltransferase activity measured at either 2, 12, 18, 24, or 48 h after the beginning of the 2 h exposure. There was no significant change in phenylethanolamine N-methyltransferase activity measured immediately following the 2 h pulse (fig. IV-5). However, fig. IV-5 shows that a 2 h pulse of nicotine was able to

increase phenylethanolamine N-methyltransferase activity at 18, 24, and 48 h, but not at 12 h, following exposure to the pulse, with increases comparable in magnitude to those seen with the 2 h pulse of cortisol. When a 2 h pulse of both cortisol and nicotine were given, the combination treatment increased phenylethanolamine N-methyltransferase activity at 12 h (12%), 24 h (60%) and 48 h (79%) to a significantly greater extent when compared to either treatment alone. Note that at 12 h, an increase in phenylethanolamine N-methyltransferase activity was seen only with the addition of both cortisol and nicotine, with no increases in activity seen with either treatment alone.

## Discussion

One of the major findings of the present study is that a cortisol pulse as short as 15 minutes is sufficient to elevate the activity of phenylethanolamine N-methyltransferase, the final enzyme in catecholamine biosynthesis responsible for the conversion of noradrenaline to adrenaline. Previously, it had been thought that the regulation of this enzyme by glucocorticoids was permissive, with corticosteroids regulating steady-state levels of phenylethanolamine N-methyltransferase (Wurtman and Axelrod, 1966; Fuller and Hunt, 1967; Thoenen et al., 1970; Ciaranello and Black, 1971). Administration of high doses of glucocorticoids to the intact rat were unable to increase phenylethanolamine N-methyltransferase activity above basal levels (Ciaranello and Black, 1971; Wurtman et al., 1967), with further increases in phenylethanolamine N-methyltransferase activity above basal levels thought to be mediated by elevated splanchnic nerve activity (Ciaranello, 1980; Thoenen et al., 1970). However, in the study where glucocorticoids were administered to intact rats (Wurtman et al., 1967), glucocorticoid concentrations in plasma and glucocorticoid content in adrenal homogenate were actually decreased (due to suppression of adrenocorticotropic hormone secretion) resulting in a lack of an increase in phenylethanolamine N-methyltransferase activity. Several studies have subsequently

shown that adrenal phenylethanolamine N-methyltransferase can be increased in intact animals following long-term treatments which increase circulating glucocorticoids (Vernikos-Danellis et al., 1968; Kvetnansky et al., 1970; Simonyi et al., 1985). In cultures of adrenal medullary cells, several groups have shown a further increase in phenylethanolamine N-methyltransferase activity and/or mRNA levels in response to continuous exposure to glucocorticoids, with at least 18 h to 2 days necessary to see any changes (Hersey and DiStefano, 1979; Kelner and Pollard, 1985; Wan and Livett, 1989; Stachowiak et al., 1990a).

The long time of exposure required for glucocorticoids to increase phenylethanolamine N-methyltransferase in all previous in vivo and in vitro experiments had led to the notion that glucocorticoids were only involved in long-term regulation of the enzyme, playing little role in more acute regulation. However, we have provided new evidence that continuous exposure of adrenomedullary cells to glucocorticoids for a prolonged period does not seem to be required for regulation of phenylethanolamine N-methyltransferase activity. Our results show that a very short pulse of cortisol (15 minutes) increases adrenal medullary cell phenylethanolamine N-methyltransferase activity 2 days after the beginning of the pulse.

There appeared to be some variation in the absolute increase in phenylethanolamine N-methyltransferase activity following exposure to various lengths of cortisol pulses, from 15 min to 48 h (fig. IV-1). It was possible that the observed pattern of increase in phenylethanolamine N-methyltransferase activity could be due to different levels of catecholamines (mainly adrenaline) in the cell lysate, interfering with the in vitro assay of phenylethanolamine N-methyltransferase. Fuller and Hunt (1967) have shown that adrenaline can inhibit the activity of phenylethanolamine N-methyltransferase in vitro and have suggested that the enzyme may be modulated in vivo via such end product inhibition. Therefore, we considered the possibility that increased adrenaline levels in the cell lysate could affect the activity of phenylethanolamine N-methyltransferase measured

in vitro. In the present study, the cell lysate prepared from control cultures contained from 3-42 μM adrenaline, with typical values in the 12-20 μM range, and addition of 40 μM adrenaline to control enzyme assay mixtures had no effect on the activity of phenylethanolamine N-methyltransferase (unpublished observations). Addition of a high concentration of adrenaline (140 μM) was able to significantly ($P<0.001$) inhibit both control and cortisol-stimulated phenylethanolamine N-methyltransferase activity by 31.6 ± 0.8% (n=14) and 33.1 ± 0.8% (n=16) respectively. Had such levels been reached in cortisol-treated cells and caused an inhibition of phenylethanolamine N-methyltransferase activity, the measured increase in activity in response to a cortisol pulse would actually have been underestimated by approximately 30%. However, no correlation was found between the levels of adrenaline in the cell lysate of cortisol-treated cells (in fig. IV-1) and the percentage increase in enzyme activity following pulses of various lengths (unpublished observations). The abovementioned results suggest that the variations in enzyme activity were not an artefact due to interference by adrenaline in the in vitro assay. It is, however, a possibility that the activity of phenylethanolamine N-methyltransferase in the intact cells may have been modulated by variations in catecholamine content of the cells at some point during the 2 day incubation of the intact cells, prior to the in vitro assay of enzyme activity.

With continuous exposure of adrenomedullary cells to glucocorticoids, a necessary time period (18-24 h) of glucocorticoid exposure is required to see increases in the activity of phenylethanolamine N-methyltransferase, with shorter times of continuous exposure being ineffective (Hersey and DiStefano, 1979; Kelner and Pollard, 1985). In this study, we investigated the requirement for a lag period following a 2 h pulse of cortisol and found that at least 18 h is needed to see increases in phenylethanolamine N-methyltransferase activity. The requirement of a lag period for glucocorticoid regulation of phenylethanolamine N-methyltransferase activity appears to be due to the mechanisms by which glucocorticoids increase the activity of this enzyme; these include effects on both

synthesis and degradation of the enzyme (Wurtman and Axelrod, 1966; Ciaranello, 1978; Berenbeim et al., 1979; Wong et al., 1985; Stachowiak et al., 1988, 1990a; Jiang et al., 1989; Wan and Livett, 1989). The long time course needed to observe an increase in phenylethanolamine N-methyltransferase activity would suggest that glucocorticoids may play a role not in the initial acute stress response itself, but rather in the recovery from acute stress, promoting catecholamine replenishment following stress-induced depletion.

There is evidence that glucocorticoids regulate phenylethanolamine N-methyltransferase activity in the adrenal medulla by acting via glucocorticoid receptors. Using cultured adrenal medullary cells, our laboratory (Betito et al., 1992) and others (Kelner and Pollard, 1985; Nawata et al., 1985) have demonstrated the presence of high affinity glucocorticoid receptors. These receptors have been immunocytochemically localized in phenylethanolamine N-methyltransferase immunoreactive cells in perfused rat adrenal slices (Ceccatelli et al., 1989). In addition, a functional glucocorticoid response element has been identified on the rat phenylethanolamine N-methyltransferase gene (Ross et al., 1990), and consensus sequences for glucocorticoid response elements have been localized in bovine and human phenylethanolamine N-methyltransferase genes (Baetge et al., 1988; Batter et al., 1988). Using the glucocorticoid receptor antagonist RU 38486 in adrenal medullary cells, Wan and Livett (1989) blocked the glucocorticoid-induced increase in phenylethanolamine N-methyltransferase mRNA. In our laboratory, RU 38486 was able to block phenylethanolamine N-methyltransferase activity increased by both continuous exposure to cortisol (Betito et al., 1992 and this study), and by a 2 h pulse of cortisol (this study), suggesting that cortisol regulation of phenylethanolamine N-methyltransferase activity and/or mRNA is glucocorticoid receptor-mediated, whether exposure is continuous or short-term.

Our experiments examining the concentrations of cortisol required to increase phenylethanolamine N-methyltransferase activity, either with continuous exposure (Betito et al., 1992) or short pulses, suggest that lower concentrations may play a permissive role

in maintaining phenylethanolamine N-methyltransferase levels, while pulses of higher concentration of glucocorticoids (such as released during stress) may produce more acute dynamic changes in phenylethanolamine N-methyltransferase activity. In the adrenal medulla, glucocorticoid concentrations are approximately 100-fold higher than in the general circulation (Kitay, 1961). Several groups have provided evidence from measurements made from adrenal venous blood of anesthetized rats (Kitay, 1961; Peytremann et al., 1973; Jones et al., 1977) or from rat adrenomedullary tissue (Guidotti and Costa, 1974) for adrenal medullary glucocorticoid concentrations in the range of 0.6 to 10 x $10^{-5}$ M. Therefore, under basal conditions in an awake animal, glucocorticoid concentrations in the adrenal medulla may in fact be slightly lower than the above mentioned range, rising to somewhere in the $10^{-4}$ M range following a stressful event. In the present study, a pulse of moderate to high concentrations of cortisol ($10^{-6}$, $10^{-5}$ and $10^{-4}$ M) was shown to increase phenylethanolamine N-methyltransferase activity 2 days later, with pulses of lower concentrations of cortisol being ineffective. By contrast, when continuous glucocorticoid exposure (18-72 h) is used, we (Betito et al., 1992) and others (Kelner and Pollard, 1985) have shown an increase in phenylethanolamine N-methyltransferase activity in adrenal medullary cells in response to concentrations as low as $10^{-8}$ M. In addition, our laboratory has shown a loss in soluble [$^3$H]dexamethasone binding (glucocorticoid receptor translocation) following exposure to a 45 minute pulse of cortisol as low as 5 x $10^{-8}$ M, as well as an increase in nuclear uptake of [$^3$H]dexamethasone in this low glucocorticoid concentration range. These findings, taken together, suggest that although lower concentrations of cortisol produce short-term dynamic changes in the hormone-receptor signal (i.e. glucocorticoid receptor translocation), adrenal medullary cells require longer term exposure to the low glucocorticoid concentrations for phenylethanolamine N-methyltransferase activity to be increased. This may reflect the permissive effect of glucocorticoids on the regulation of basal phenylethanolamine N-methyltransferase activity. The higher concentrations of a

brief cortisol pulse needed to increase the enzyme may reflect the physiological situation of acute stress, where cortisol levels rise above basal levels for a short period of time (1 to 2 h).

The experiments in this study were carried out on cells maintained in medium containing 10% fetal bovine serum (control medium), which has very low (nanomolar) levels of glucocorticoids (GIBCO, personal communication; unpublished observations). In vivo, the medulla is exposed to micromolar levels of glucocorticoids basally, rising at least 10-fold following stress (see above). Thus in order to mimic the physiological situation, adrenal medullary cells were preincubated with $10^{-6}$ M cortisol for 4 days, pulsed with $10^{-4}$ M cortisol, and phenylethanolamine N-methyltransferase activity measured 2 days later. Cells pretreated with $10^{-6}$ M cortisol and then exposed to a 2 h pulse of $10^{-4}$ M cortisol showed an increase in phenylethanolamine N-methyltransferase activity 2 days later of the same magnitude (30-40%) as that seen in cells maintained in control medium and exposed to a cortisol pulse. This result indicates that a short pulse of a high concentration of cortisol (similar to what occurs during an acute stress) is still effective in increasing phenylethanolamine N-methyltransferase activity above that maintained by a background or basal level of cortisol.

Although an attempt was made to mimic the in vivo situation with respect to cortisol exposure following a stressor, it must be noted that the medulla is exposed to splanchnic nerve contents (acetylcholine and peptides) which may also modulate the activity of phenylethanolamine N-methyltransferase. A variety of neurotransmitters and neuromodulators have been shown to regulate phenylethanolamine N-methyltransferase activity and/or mRNA levels, such as acetylcholine acting at nicotinic receptors (Evinger et al., 1988; Stachowiak et al., 1990a), histamine (Evinger et al., 1988), angiotensin (Stachowiak et al., 1990b), and nerve growth factor (Acheson et al., 1984). Continuous exposure of adrenal medullary cells to nicotine results in no change (Wan et al., 1991) or increases (Evinger et al., 1988; Stachowiak et al., 1990a) in phenylethanolamine N-

methyltransferase mRNA. In vivo, exposure to acetylcholine following acute stress is of short duration. We therefore examined whether a 2 h pulse of nicotine could regulate the activity of phenylethanolamine N-methyltransferase. Immediately after the nicotine pulse, there was no change in the activity of the enzyme, whereas 18 - 48 h following the pulse, phenylethanolamine N-methyltransferase activity increased. This is the first report, to our knowledge, that short-term exposure to nicotine can regulate phenylethanolamine N-methyltransferase activity in adrenal medullary cells. Since acetylcholine has been shown to release corticosteroids from perfused rat adrenals (Porter et al., 1988), we considered the possibility that nicotine may be regulating the activity of phenylethanolamine N-methyltransferase by causing the release of glucocorticoids from contaminating cortical cells present in adrenal medullary cell cultures. However, in bovine tissue, acetylcholine stimulation of glucocorticoid secretion appears to be mediated via muscarinic receptor activation (Hadjian et al., 1982), therefore stimulation of glucocorticoid secretion cannot account for the increase in phenylethanolamine N-methyltransferase activity produced by nicotine.

The presence of the combination of nicotine and cortisol was able to produce a greater increase in phenylethanolamine N-methyltransferase activity than that produced by either agent alone, suggesting that the co-ordinate acute activation of both neural (nicotinic) and hormonal (glucocorticoid) influences may serve to regulate adrenomedullary phenylethanolamine N-methyltransferase. Since the combination-induced increase does not appear to be completely additive, cortisol and nicotine may be increasing phenylethanolamine N-methyltransferase activity, in part, via a similar mechanism. One possibility is regulation of phenylethanolamine N-methyltransferase activity via increasing mRNA levels, which occurs in response to both cortisol (Wan and Livett, 1989; Stachowiak et al., 1990a) and nicotine (Evinger et al., 1988; Stachowiak et al., 1990a). It is interesting to note that the presence of the combination of cortisol and nicotine (in comparison to either agent alone) seems to shift the required lag period to

observe an increase in phenylethanolamine N-methyltransferase from 18 h to 12 h. However, in vivo, factors released from the adrenal cortex (other than glucocorticoids) or the splanchnic nerve (other than acetylcholine) may also influence the time course of regulation of phenylethanolamine N-methyltransferase activity, a situation not reproduced in this in vitro preparation. We have recently provided in vivo evidence in the rat for increases in adrenal phenylethanolamine N-methyltransferase activity 18-24 h following a 20 minute restraint stress, where increases in plasma glucocorticoids are maintained for approximately 1 h (Betito, Mitchell, Bhatnagar, Boksa, and Meaney, submitted), suggesting that phenylethanolamine N-methyltransferase activity can be elevated above basal levels by short-term exposure to high concentrations of glucocorticoids in vivo, as well as in vitro.

In conclusion, the present study demonstrates the in vitro regulation of adrenomedullary phenylethanolamine N-methyltransferase activity by short-term exposure to glucocorticoids, or to nicotine, with a required lag period to observe increases in enzyme activity. A single glucocorticoid exposure of 30 min increases phenylethanolamine N-methyltransferase activity 18 h later, but not at earlier times. Brief (2 h) exposure to moderate to high concentrations of cortisol increased phenylethanolamine N-methyltransferase activity, with lower concentrations being ineffective. [However, continuous long-term exposure to the lower cortisol concentrations can increase phenylethanolamine N-methyltransferase activity (Betito et al., 1992)]. These results suggest that short-term dynamic increases in glucocorticoids to high levels, such as encountered by the adrenal medulla during an acute stress, can cause increases in phenylethanolamine N-methyltransferase activity, while lower (basal) levels of glucocorticoids function to maintain steady-state phenylethanolamine N-methyltransferase levels on a longer-term basis.

**Figure IV-1. A pulse as short as 15 minutes is sufficient to increase phenylethanolamine N-methyltransferase activity 2 days later.** Bovine adrenal medullary cells were treated with cortisol [$10^{-5}$ M (open bar) or $10^{-4}$ M (filled bar)] for various times, the medium replaced with control medium and phenylethanolamine N-methyltransferase activity measured 2 days following the beginning of cortisol exposure. Data are expressed as a percent control (untreated cultures), each bar representing the mean (±S.E.M.) value for 14-30 plates from 2 to 3 cell culture preparations except for the 24 h time point (9-11 plates from 1 preparation) (significantly different from control untreated cultures at *P<0.05, **P<0.02, ***P<0.005).

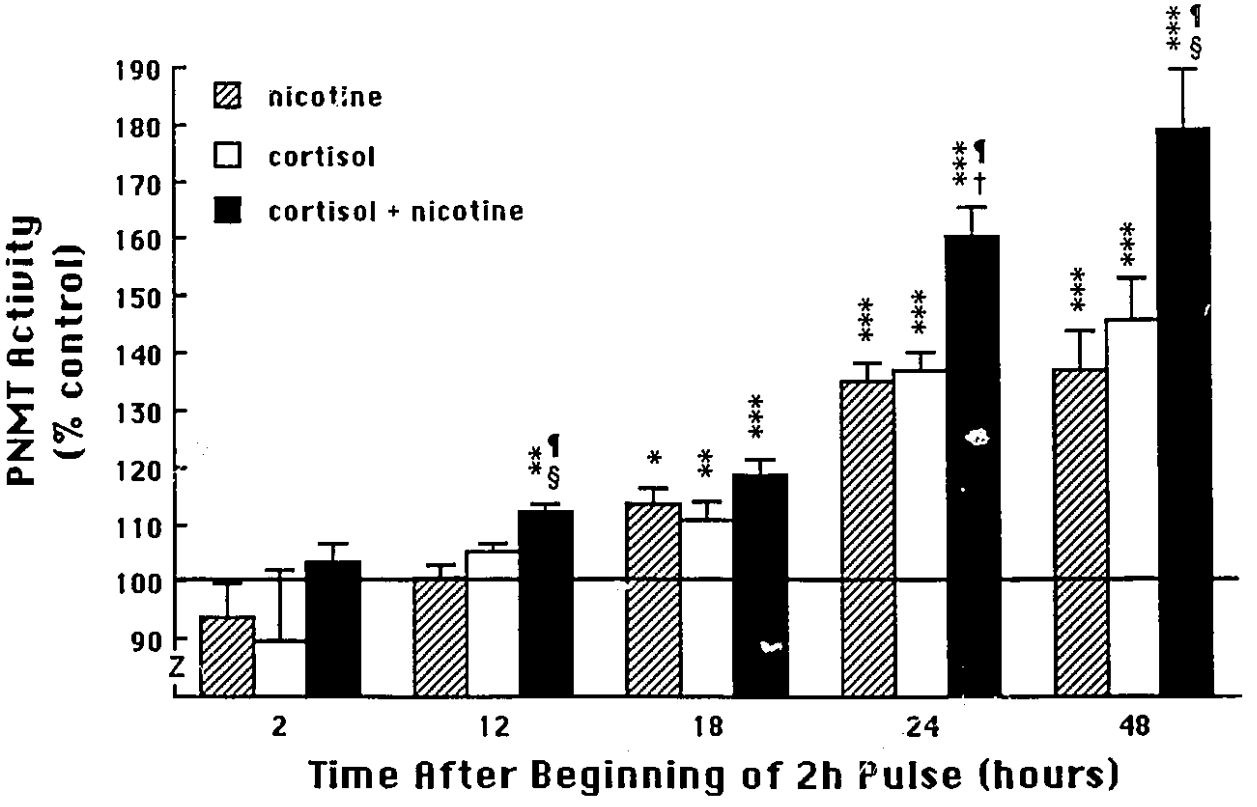
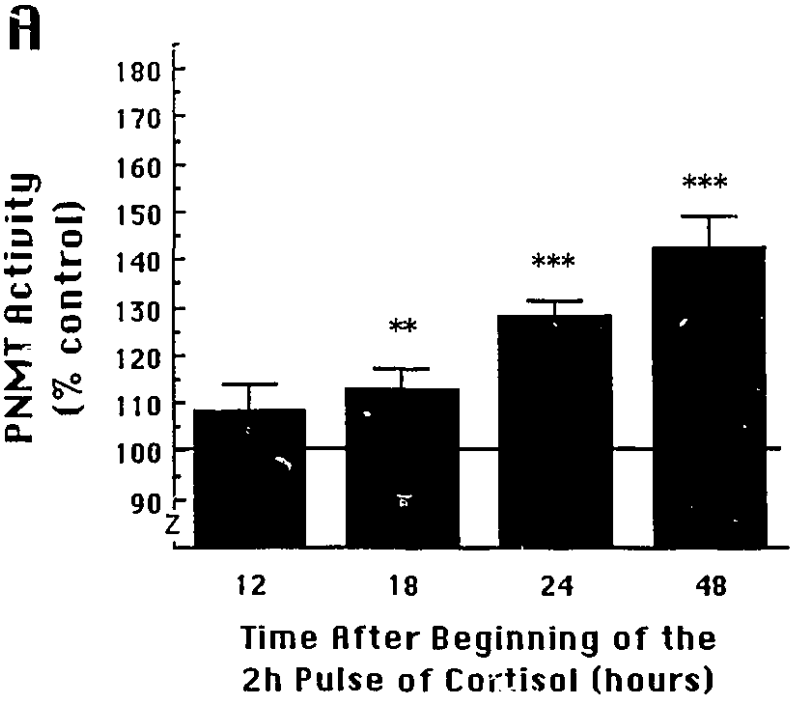

PNMT Activity
(% control)
190
180
170
160
150
140
130
120
110
100
90
nicotine
cortisol
cortisol + nicotine
2
12
18
24
48
Time After Beginning of 2h Pulse (hours)

**Figure IV-2. A lag period is required for a pulse of cortisol to increase phenylethanolamine N-methyltransferase activity.** Adrenal medullary cells were exposed to a 2 h pulse (A) or 30 minute pulse (B) of $10^{-4}$ M cortisol, the cortisol removed and replaced with control medium, and the cells assayed for phenylethanolamine N-methyltransferase activity at either 12, 18, 24, or 48 h after the beginning of the 2 h (or 30 min) cortisol pulse. In (A), each bar represents the mean (±S.E.M.) value for 40-56 plates from 3 to 6 cell culture preparations. In (B), each bar represents the mean (±S.E.M.) value for 20-21plates from 3 cell culture preparations. (significantly different from control untreated cultures at *P<0.05, **P<0.01, ***P<0.0005).


A
PNMT Activity
(% control)
180
170
160
150
140
130
120
110
100
90
**
***
***
12
18
24
48
Time After Beginning of the
2h Pulse of Cortisol (hours)

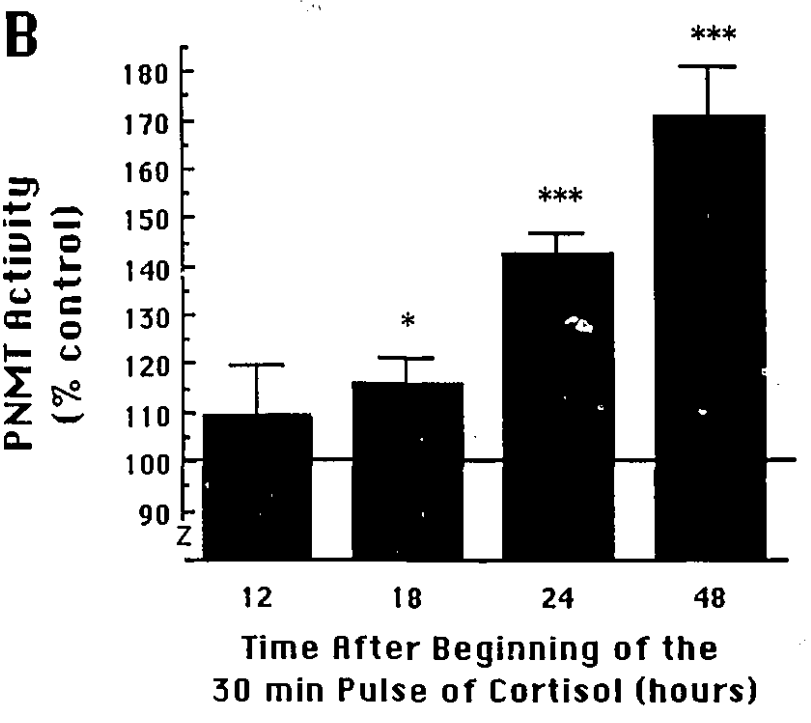

B
PNMT Activity
(% control)
180
170
160
150
140
130
120
110
100
90
*
***
***
12
18
24
48
Time After Beginning of the
30 min Pulse of Cortisol (hours)

**Figure IV-3. A pulse of various concentrations of cortisol increases phenylethanolamine N-methyltransferase activity.** Cells were exposed to various concentrations of cortisol ($10^{-9}$ to $10^{-4}$ M) for 2 h, the medium replaced and phenylethanolamine N-methyltransferase activity assayed 2 days later. Each bar represents the mean (±S.E.M.) value for 22-38 plates from 3 ($10^{-4}$ and $10^{-5}$ M), 8 ($10^{-6}$ and $10^{-7}$ M), or 5 ($10^{-8}$ and $10^{-9}$ M) cell culture preparations (significantly different from control untreated cultures at *P<0.05, **P<0.002).

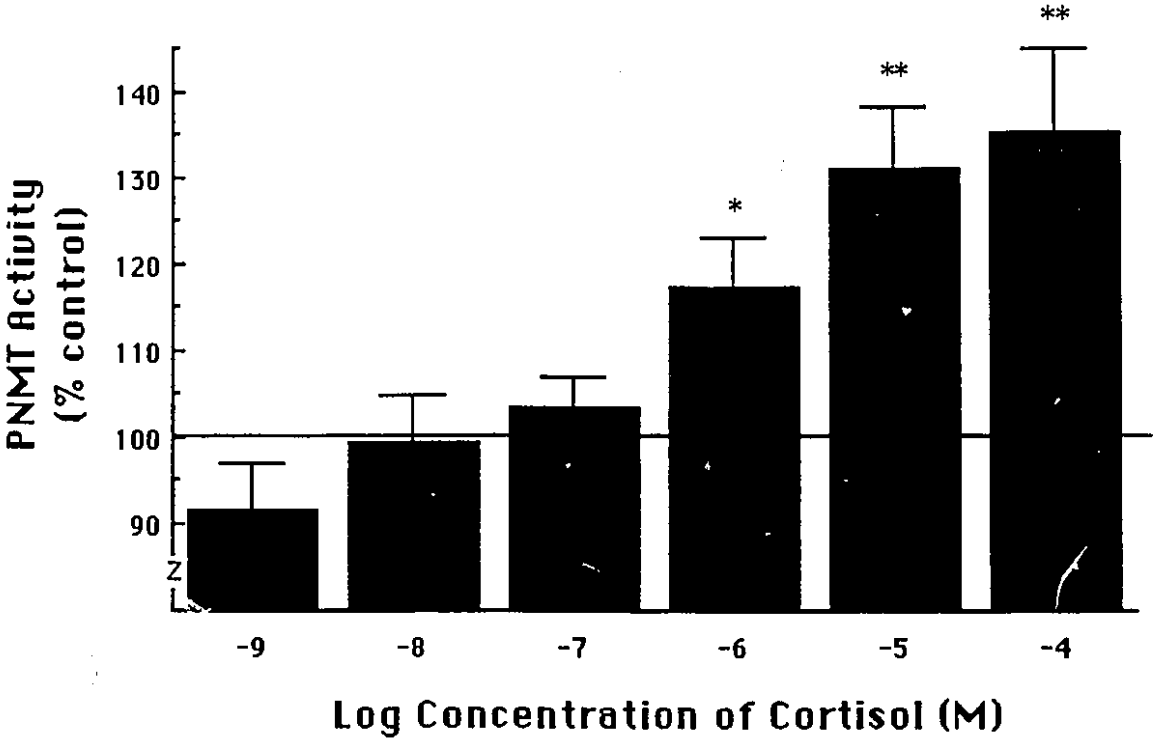

PNMT Activity
(% control)
140
130
120
110
100
90
*
**
**
-9
-8
-7
-6
-5
-4
Log Concentration of Cortisol (M)

**Figure IV-4. A 2 h pulse of cortisol increases phenylethanolamine N-methyltransferase activity in adrenal medullary cells that had been pretreated with $10^{-6}$ M cortisol for 4 days.** Adrenal medullary cells were pretreated with either medium containing vehicle ("control pretreatment") or medium containing added cortisol ($10^{-6}$ M) for 4 days ["4 day cortisol ($10^{-6}$ M) pretreatment"]. The medium was removed from all culture plates, and the plates were pulsed for 2 h with either control medium (open bars for each pretreatment) or medium containing $10^{-4}$ M cortisol (filled bars for each pretreatment). All plates then received control medium for 2 days, the cells were scraped and phenylethanolamine N-methyltransferase activity measured. Each bar represents the mean (± S.E.M.) value for 25-30 plates from 2 cell culture preparations [*$P<0.03$ compared to control pretreatment, 2 h vehicle pulse (open bar on left hand side of graph); **$P<0.001$ compared to control pretreatment, 2 h vehicle pulse (open bar on left hand side of graph); §$P<0.001$ compared to 4 day cortisol ($10^{-6}$ M) pretreatment, 2 h vehicle pulse (open bar on right hand side of graph)].

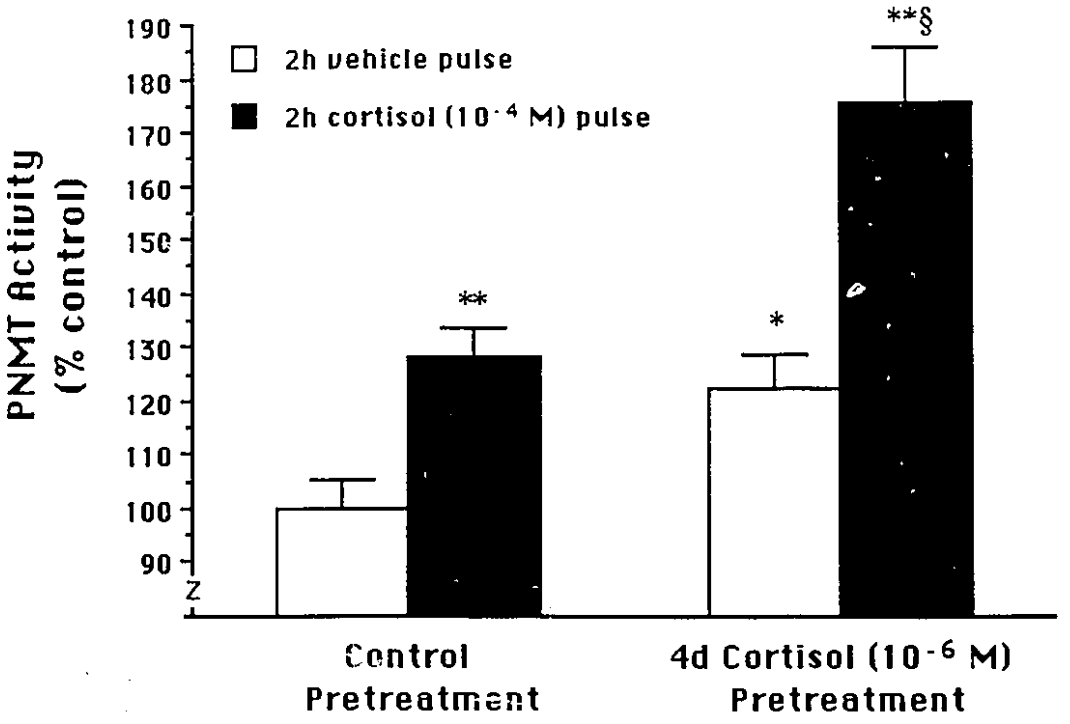

PNMT Activity
(% control)
190
180
170
160
150
140
130
120
110
100
90
2h vehicle pulse
2h cortisol (10⁻⁴ M) pulse
**
*
**§
Control
Pretreatment
4d Cortisol (10⁻⁶ M)
Pretreatment

**Figure IV-5. A 2 h pulse of nicotine increases phenylethanolamine N-methyltransferase activity following a required lag period.** Adrenal medullary cells were treated with either nicotine ($10^{-5}$ M, hatched bar), cortisol ($10^{-4}$ M, open bar), or both (filled bar) for 2 h, the medium replaced with control medium and phenylethanolamine N-methyltransferase activity assayed either 2, 12, 18, 24, or 48 h following the beginning of the 2 h drug exposure. Each bar represents the mean (±S.E.M.) of 31-37 determinations from 3 cell culture preparations except for the 2 and 12 h time points which were from 8-12 determinations from 1 preparation (*P<0.01,**P<0.005, ***P<0.0005 compared to control untreated cultures, †P<0.002 compared to cortisol alone, §P<0.05 compared to cortisol alone, ¶P<0.005 compared to nicotine alone).

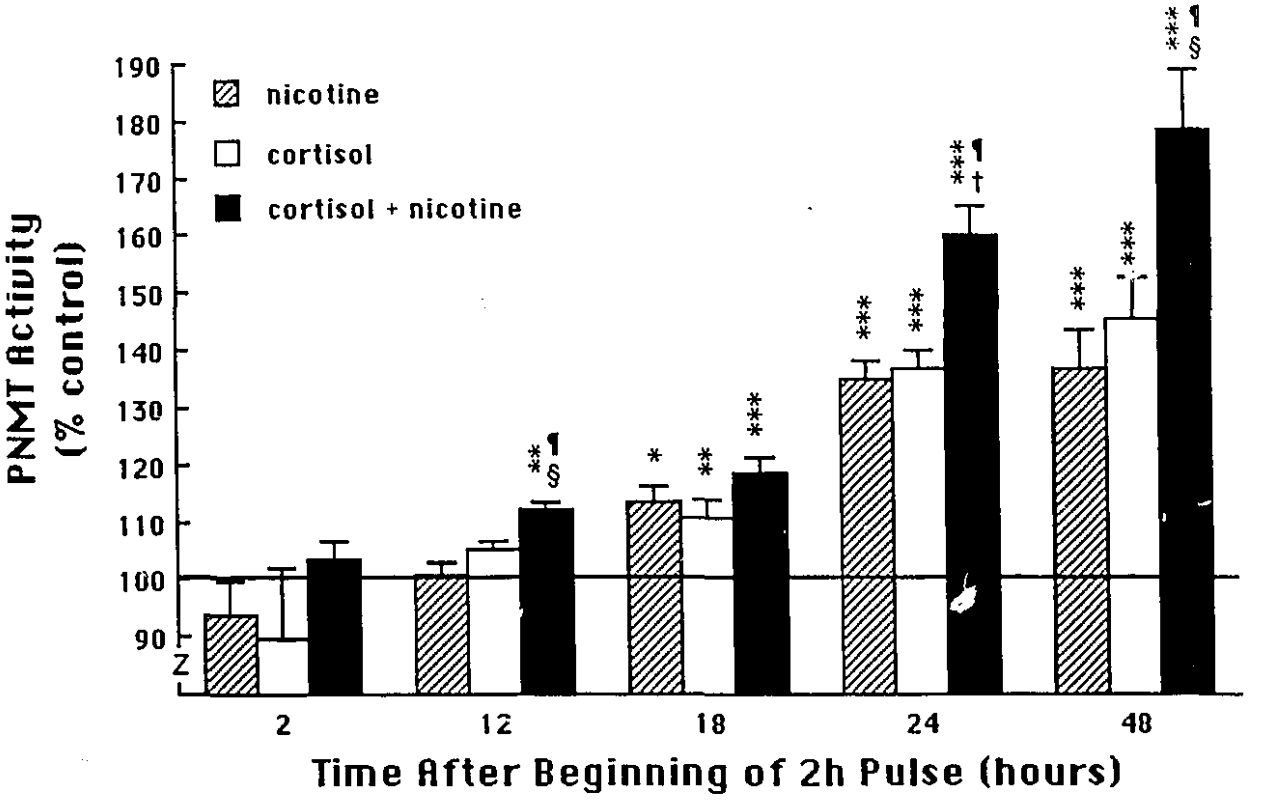

PNMT Activity
(% control)
190
180
170
160
150
140
130
120
110
100
90
nicotine
cortisol
cortisol + nicotine
2
12
18
24
48
Time After Beginning of 2h Pulse (hours)

**Table IV-1. The effect of the glucocorticoid receptor antagonist RU 38486, on phenylethanolamine N-methyltransferase activity induced by a 2 h pulse of cortisol.** Adrenal medullary cells were treated with $10^{-5}$ M cortisol in the absence or presence of $10^{-5}$ M RU 38486, either continuously for 2 days (2 day exposure) or with a 2 h pulse as in previous experiments, and phenylethanolamine N-methyltransferase activity measured 2 days following the beginning of cortisol exposure. The data are expressed as the mean (± S.E.M.) of 23-28 plates taken from 2-3 cell culture preparations ([a], $P<0.05$ compared to control untreated cultures; [b], $P<0.01$ compared to control untreated cultures; [c], $P<0.01$ compared to cortisol in the absence of RU 38486).

| | Phenylethanolamine N-methyltransferase Activity (% control) | |
|---|---|---|
| | $10^{-5}$ M cortisol | $10^{-5}$ M cortisol +$10^{-5}$ M RU 38486 |
| 2 day exposure | 152.5 ± 7.5 (28)[b] | 123.9 ± 5.5 (28)[b,c] |
| 2 h pulse | 124.5 ± 9.0 (24)[a] | 88.3 ± 4.6 (23)[c] |

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# Chapter V. Glucocorticoid Regulation of the Adrenomedullary Catecholaminergic System Following Mild, Acute Stress

## Abstract

The time course of regulation of rat adrenomedullary PNMT and TH activity, and catecholamine content was studied following a single episode of 20 minute restraint stress. Significant increases in PNMT and TH activity were observed 18h following the beginning of the stress. In addition, significant increases in the adrenal content of adrenaline, noradrenaline and dopamine were observed at 18h. The time course of acute stress-induced regulation of PNMT and TH was examined for the influence of hormonal input. Suppression of endogenous corticosterone with dexamethasone delayed the stress-induced increase in activity of PNMT but not TH. The present study indicates that increases in catecholamine biosynthesis can be observed following a single episode of mild acute stress. In addition, glucocorticoids appear to be important in the time course of the stress-induced increase in PNMT but not TH activity.

## Introduction

Secretion of catecholamines (CAs) from the adrenal medulla occurs in response to the release of acetylcholine (ACh) from the splanchnic nerve. During stress, the nerve increases firing at the adrenal medulla to release CAs, and pituitary adrenocorticotropic hormone (ACTH) causes the release of corticosterone (B) from the adrenal cortex into the general circulation. As a result of the close proximity of the adrenal medulla to the cortex, the medullary chromaffin cells are exposed to high levels of glucocorticoids (GCs), under both basal and stress conditions ($10^{-6}$ to $10^{-4}$ M; Kitay, 1961; Peytremann et al., 1973; Guidotti and Costa, 1974; Jones et al., 1977). Therefore, the encapsulation of the medulla by the cortex provides a unique oppurtunity for an interaction of the endocrine adrenocortical GC system and the neural crest-derived adrenomedullary CA system.

The regulation of adrenal CA biosynthesis occurs via both adrenocorticosteroid and neural influences. Hormonal regulation of the activity of tyrosine hydroxylase (TH; the rate limiting step in CA biosynthesis; Levitt 1965), and of phenylethanolamine N-methyltransferase (PNMT; which catalyses the conversion of noradrenaline to adrenaline; Axelrod 1962), has been reported. Hypophysectomy or chronic suppression of endogenous B has been shown to reduce both TH and PNMT activity and/or mRNA (Wurtman and Axelrod, 1965; 1966; Wurtman, 1966; Mueller et al., 1970; Stachowiak et al., 1988; Jiang et al., 1989; Mitchell and Vulliet, 1985; Wurtman et al., 1966). In vitro studies using adrenal medullary cells in culture have demonstrated a GC-induced increase in TH and/or PNMT activity or mRNA levels (Hersey and DiStefano, 1979; Kelner and Pollard, 1985; Wan and Livett, 1989; Stachowiak et al., 1990; Ross et al., 1990; Betito et al., 1992). Therefore, these studies demonstrate a hormonal regulation of both PNMT and TH.

The time course of regulation of TH and PNMT depends on a variety of factors. A number of studies have shown an increase in TH activity and/or mRNA by short-term drug treatment or acute stress (for review see Zigmond et al., 1989). For PNMT activity,

regulation was previously thought to be homeostatic in nature, with increases in intact rats observed only following chronic manipulations of the hormonal or neural environment. However, there are a few studies which illustrate an increase in PNMT activity following acute manipulations such as stress. Significant increases have been observed following acute (1-2.5h) stressors such as cold, forced swimming or immobilization (Kvetnansky et al., 1970; Kvetnansky, 1973; Hoffman et al., 1975; Bhagat and Horenstein, 1976). The increase in PNMT activity is apparent 6-32h post-stress and can be abolished by hypophysectomy (Kvetnansky, 1973; Bhagat and Horenstein, 1976). Therefore, these studies seem to refute the long-held notion that regulation of PNMT activity occurs only under chronic stress conditions. However, these studies used acute stressors of 1-2h duration, and generally chose one time point following stress in which to measure enzyme activity (usually 48h later). No study has examined the regulation of CA biosynthesis in response to a more mild stressor (such as restraint stress) of short (<1h) duration, nor has a study systematically investigated the time course required to see increases in CA biosynthetic enzymes following an acute stress.

In the present study, our first objective was to investigate the time course of regulation of PNMT and TH activity and CA content in response to short duration exposure of the whole animal to a single episode of mild stress. In vitro, we have previously shown that exposure of bovine adrenal medullary cells to a short-term (15 min - 2h) pulse of GCs elevates the activity of PNMT measured a few days later, and that the cells require at least 18h in order to increase the activity of this enzyme in response to a short pulse (Betito et al., 1992; 1993). The second objective was to examine the importance of hormonal input on the time course of acute stress-induced regulation of PNMT and TH. Our in vitro studies had shown that the activity of PNMT can be increased 18-24h after the onset of a short (2h) pulse of GCs (Betito et al., 1993). Therefore, at least in vitro, short-term exposure of the adrenal medulla to GCs is sufficient to regulate this enzyme following a required time delay. In the present study,

we examined whether hormonal influence is necessary to increase CA biosynthesis following a mild, acute stress of short duration (20 min restraint), and studied in detail the time requirements needed to increase activity of CA biosynthetic enzymes.

## Experimental Procedures

Male Long-Evans rats (250g-400g; Charles Rivers Canada, St. Constant, Quebec), were maintained on a 12h light-dark cycle (08:00h lights on) with food available ad libitum. Rats were subjected to a 20 minute restraint stress, using a lucite tube with a closed end. For time course experiments, blood was drawn from the tail vein (≈100 μL) at various intervals. For other experiments, trunk blood was collected following decapitation. Blood was collected into iced tubes containing EDTA and aprotonin (TRASYLOL; Miles Canada Inc.), centrifuged at 3000*g* for 10 min, and the supernatant used for plasma B determination by radioimmunoassay (see below). All animals were sacrificed by rapid decapitation between 10 am and 2 pm. Stress testing was performed during the light cycle (12:30-17:00h) except for the 36h sacrifice time points in one set of experiments (DEX suppression experiments - see below), where the stressor was given at 22:00h. For all experiments, adrenal glands were collected, cleaned free of fat and rapidly frozen on dry ice and used for enzyme activity assays (see below).

## Measurement of Plasma B and ACTH Concentrations

The concentration of B was measured in plasma prepared from samples of either trunk blood (for B concentration at time of sacrifice) or tail vein blood (at time of stress and in the stress recovery experiments). An aliquot (10 μL) of the plasma was extracted into 990 μL of absolute ethanol, the samples centrifuged at 1800*g*, and 100 μL of the supernatant in duplicate freeze-dried and used for B radioimmunoassay, with a highly specific B antiserum (B3-163, Endocrine Sciences, Tarzana, CA), as previously described (Krey et al., 1975). The detection limit of the assay was 5 pg/mL. ACTH was

measured by radioimmunoassay as previously described (Walker et al., 1990) using 50 μL aliquots of plasma. Briefly, unextracted samples were incubated at 4°C with tracer ([$^{125}$I]-ACTH, 5000 cpm/tube; Incstar, Stillwater, MN) and ACTH antiserum (final dilution of 1:120,000; IgG Corp., Nashville, TN). After 72h of incubation, precipitation serum (Antibodies Inc., Davis, CA) was added and bound peptide obtained by centrifugation at 5000*g* for 45 min. The detection limit of the assay was 10 pg/mL. Intra-assay coefficients of variation for the plasma B and ACTH assays were 8.9 and 6.0% respectively. The inter-assay coefficients of variation were 10.9% for both assays.

**Measurement of PNMT activity**

Right adrenal glands were homogenized in water (1 mL/gland) using a Dounce homogenizer, and subjected to a freeze-thaw cycle (-80°C). Samples were then centrifuged at 12 800*g* for 10 min, and aliquots (50 μL) of the supernatant were assayed for PNMT activity as described by Pollard et al. (1979). The assay is based on catalysis of the methylation of substrate (phenylethanolamine, 1.8 mM) by [6-methyl-$^{3}$H]-S adenosyl methionine (10 μM) and extraction of tritium labelled methyl product into toluene/isoamyl alcohol. Non-specific methyltransferase activity, assessed with parallel standards containing substrate and methyl donor, was subtracted from all samples.

**Measurement of TH Activity**

Left adrenal glands were homogenized in 600 μL Trizma (5 mM) containing Triton X-100 (1 mg/mL), centrifuged at 12 800*g* for 10 min, and aliquots (120 μL) of the supernatant assayed for TH activity according to the method of Nagatsu et al. (1964) as modified by Quik and Sourkes (1976). The incubation mixture in a final volume of 200 μL, and at a pH of 6.5, contained the following components: 40 μL of a solution containing Trizma acetate (50 mM), 560 U catalase, and brocresine (0.5 mM); 120 μL of the adrenal homogenate; and 20 μL of a solution containing nonradioactive tyrosine (0.92

mM) and [$^3$H]-tyrosine (500 000 disintegrations/ minute). These components were preincubated at 37°C and the reaction started with 20 µL with a solution containing ascorbic acid (24.6 mM) and $DMPH_4$ (10 mM). The reaction was stopped after 15 min with 40 µL of a 25% trichloroacetic acid (TCA) solution. Precipitated protein was pelleted by centrifugation at 12 800*g* for 10 min and 100 µL of the supernatant applied to a Dowex 50W-X8 column (0.5x2.0 cm), preequilibrated with 2 mL of TCA (pH 1.8). The column was washed with 900 µL TCA (pH 1.8) and radioactivity in the effluent (separated into two aliquots) measured using 5 mL Ecolite scintillation fluid at 50% efficiency.

### Measurement of CAs

CAs were measured by HPLC with electrochemical detection, using epinine as the internal standard. An aliquot of adrenal homogenate in HPLC-grade water (50 µL) was used for alumina (10 mg) extraction of CAs. The conditions used for HPLC separation and detection of adrenaline (A), noradrenaline (NA), and dopamine (DA) were as described by Boksa (1990) except that the mobile phase contained 100 mg/ml sodium octyl sulfate.

### Statistical Analyses

Data were analyzed using one-way analysis of variance (time course following restraint stress experiment) or two way analysis of variance (DEX experiments) with post-hoc Dunnet's comparisons ($p<0.05$). Comparisons for the effect of drug treatment at a particular time point were performed using the F-test for simple main effects (alpha level taken to be significant at $p<0.05$/number of comparisons).

## Results

### The regulation of adrenal PNMT and TH activity and CA content at various time points following a single 20 minute restraint stress

In the present experiment, we sought to examine the effects of a single period of stress (20 min restraint) on adrenal CA biosynthetic enzymes using a detailed time course, measuring plasma B and ACTH, adrenal activity of PNMT and TH, and adrenal content of adrenaline (A), noradrenaline (NA) and dopamine (DA), at various time points following the end of the restraint. Rats were sacrificed by rapid decapitation at various intervals following restraint stress, i.e. immediately following the stressor (time 0) and at 2, 6, 18, 24, 48, and 96h after termination of the stressor, and adrenal glands collected and rapidly frozen. Trunk blood was collected to measure plasma B and ACTH levels (Table V-1). Significant increases ($p<.01$) in both ACTH and B were observed immediately following the stressor (time 0), and at 2h for B, with return to basal values thereafter.

The activities of PNMT and TH were measured in adrenal homogenates at the same time points as above (PNMT was taken from the right adrenal, TH from the left in the same animal). Figure V-1A shows that following a single episode of acute stress, a significant increase in adrenal PNMT activity is observed only at 18h after the end of the 20 min restraint. No significant increases were observed at either earlier (2h, 6h) or later (24h, 48h, 96h) time points following the stressor. Similar findings were observed for TH activity (Figure V-1B).

Adrenal content of CAs were measured in homogenates of right adrenal glands at the same time points as above. Figure V-2 shows that following a single episode of acute stress, a significant increase in DA is observed immediately following restraint stress (0h

in Figure V-2A), as well as at 18h. In addition, significant increases in adrenal content of A and NA were observed at 18h but at no other time points (Figure V-2B and V-2C).

**The effect of DEX suppression of endogenous B secretion on PNMT and TH activity**

The activities of PNMT and TH are reduced by hypophysectomy, suggesting a role for GCs in the maintenance of both enzyme activities. Since hypophysectomy may have effects involved in stress physiology other than simply abolishing the source of ACTH, we isolated the effect of GCs on CA biosynthesis using DEX suppression of endogenous B secretion. Rats were injected subcutaneously with DEX (100 μg/kg; a dose that does not affect the basal activity of PNMT and TH, see below) dissolved in saline:ethanol (9:1) 3 hours prior to the 20 min restraint stress. One set of animals were used for stress recovery sampling of plasma for B to ensure suppression by the DEX treatment. Another set of animals underwent DEX or vehicle injections, were restrained for 20 min, and had blood taken at the end of the stressor (to ensure effectiveness of DEX suppression). Rats were sacrificed at 18, 24 and 36h following the beginning of the stressor. Control (unstressed) animals were injected with the vehicle or DEX 27h prior to sacrifice.

Stress-induced changes in plasma B were first examined to test the effectiveness of the DEX suppression. A significant main effect of DEX on plasma B (Figure V-3) levels were observed at all time points. Figure V-4 shows the activities of PNMT and TH at various time points following restraint, in vehicle and DEX treated animals. The control bars represent the activity of either enzyme in the adrenals of rats injected with the vehicle or DEX 27 hours prior to sacrifice. This time point verified that DEX on its own did not affect the activity of either enzyme, and was chosen to mimic the pattern of injection and sacrifice for the 24h post-stress time point set of animals. Three groups of rats (16 per group) were either injected with the vehicle or DEX, subjected to a 20 min restraint stress

3h later, and sacrificed at 18, 24 and 36h following the beginning of the stressor. Significant ($p<.005$) effects of both time and DEX administration were observed for PNMT activity (Figure V-4A). There was a significant time by DEX interaction ($p=.02$), indicating that vehicle- and DEX-treated rats differed over time. In vehicle-treated rats, post-hoc tests showed a significant ($p<.05$) increase in PNMT activity at 24 and 36h. For the DEX treatment, a significant ($p<.05$) increase in PNMT activity was observed at 36h, with a simple effects measure of the effect of DEX revealing a significant difference ($p<.0001$) between the vehicle- and DEX-treated group at the 24h time point. This indicates a suppression or delay of the increase in PNMT activity in response to the 20 min restraint stress among DEX-treated animals. The activity of TH (Figure V-4B) increased similarly in both the vehicle- and DEX-treated groups (time by DEX interaction, $p=.8$; effect of time $p<.0002$) with increases observed at 18h as measured by post-hoc tests ($p<.01$ for vehicle-treated; $p<.05$ for DEX-treated rats).

## Discussion

*PNMT Activity*

The adrenal medulla releases CAs into the bloodstream during an acute stress. However, the CA stores must be replenished following the stressor. We provide evidence for the possibility of replenishment of these stores via a regulation of CA biosynthetic enzymes in response to a mild acute stress (20 min restraint). In the present study, increases in PNMT activity were observed following a single episode of acute stress (Figure V-1B). However, this increase occurred only following a specific time delay, within a window of 18-36h following the beginning of the stressor. These in vivo findings support earlier in vitro studies performed by this laboratory using cultured bovine adrenal medullary cells. We had shown that exposure of these cells for 2h to GCs increased the activity of PNMT, with a time delay of at least 18h (Betito et al., 1993).

Therefore, both our in vivo and in vitro studies suggest that PNMT can be regulated by short-term dynamic changes in the adrenal environment, but that this increase can only be observed with a required time delay.

The present study suggests that GCs at the level of the adrenal medulla, play an important role in the recovery of CA levels following stress. The GC regulation of PNMT activity was confirmed by the experiments in this study using a mild acute stressor. Injection of DEX 3h prior to restraint stress, to suppress the stress-induced release of endogenous B, delayed the increase in PNMT activity. In vehicle-treated rats, significant increases were apparent at 24 and 36h post-stress. In the DEX treated rats, the trend in the increases in enzyme activity over time was different, with significantly less PNMT activity observed at the 24h time point compared to the vehicle-treated animals. Therefore, the effect of GC secretion following an acute restraint stress appears to be to shift increases in PNMT activity to earlier time points. Had we not looked at further time points following stress (ie. >24h), we would have concluded that GCs were crucial for the increase in PNMT. However, the timing of the enzyme measurements post-stress appears to be critical since an increase in PNMT activity in DEX-treated animals was observed at 36h.

Our results suggest that the time course for increasing PNMT activity post-stress may be affected by an interaction between hormonal and neural influences. This is supported by our in vitro studies using bovine adrenal medullary cells (Betito et al., 1993). The time course of increasing PNMT activity following a 2h pulse of either cortisol or nicotine was at least 18h. However, there was a larger and earlier (12h vs 18h) increase in PNMT following a simultaneous pulse of both drugs. This suggests that the timing for increasing this enzyme is dependent on the regulatory factors present and/or an interaction between these factors. In vivo (in the present study) the absence of hormonal influence or of a hormonal/neural interaction due to DEX suppression results in a loss in the early increase in PNMT activity. The presence of both hormonal and neural

influences causes an increase in PNMT at 18-24h post-stress (i.e. Figure V-1A and vehicle in Figure V-4A) whereas the increase is later in the absence of hormonal influence (DEX treatment in Figure V-4A). Therefore, the activity of PNMT seems to be regulated both in the presence and absence of hormonal input, with different time delays apparent depending on the adrenal environment.

The findings in our DEX suppression experiment are different from those found in the 2h forced swimming stress paradigm (Bhagat and Horenstein, 1976), where hypophysectomy abolishes the stress-induced increase in PNMT activity 48h later. One major difference that could account for the discrepancy found between this and our study, is that only one time point was chosen to measure changes in enzyme activity in the study by Bhagat and Horenstein (1976). Therefore, a possible increase in activity may have been measured had a more complete time course been performed. In addition, hypophysectomy results in a number of physiological changes, removing a variety of pituitary peptides or hormones, which may regulate PNMT activity. GC regulation of PNMT activity was examined in a more direct manner in the present study, since DEX suppression isolates the effect of GCs by suppressing endogenous B production, without the need for surgical intervention. Therefore, the results we are reporting suggest that B production during the stressor can shift the time frame for increases in PNMT activity, but is not completely essential for the regulation of this activity. In the absence of B secretion, other factors (either neural or hormonal) may play a role in PNMT regulation.

*TH Activity*

Following a single episode of acute stress, TH activity increases with a similar time course as PNMT, with significant increases observed at 18h post-stress. However, for TH, there is a trend for early increases in activity (see Figure V-1B), which may be due to a transient phosphorylation of the enzyme (for review see Zigmond et al., 1989). The increase in activity observed at 18h is likely accounted for by a second mechanism,

normally occuring after a delay of 12-48h, which involves increases in the amount of TH protein, and can be blocked by RNA and protein synthesis inhibitors (Mueller et al., 1969; Thoenen et al., 1973; Zigmond et al., 1989; but see McMahon et al., 1992). Therefore, the present study confirms the ability of acute stress to increase the activity of TH within the reported time delay window.

GCs have been reported to regulate the activity of TH. In the present study, we report that GCs are not crucial for this regulation, since DEX suppression of B release did not prevent the increase in TH activity observed 18h post-stress. This finding is not surprising since the activity of TH is thought to be predominantly neurally regulated. In addition, this finding is similar to the swimming stress study of Bhagat and Horenstein (1976) where hypophysectomy did not abolish the increase in TH activity, suggesting that pituitary input on adrenal function does not include the regulation of TH. Therefore, the activity of TH can be regulated by acute restraint stress followed by a time delay, in the absence of hormonal input to the adrenal.

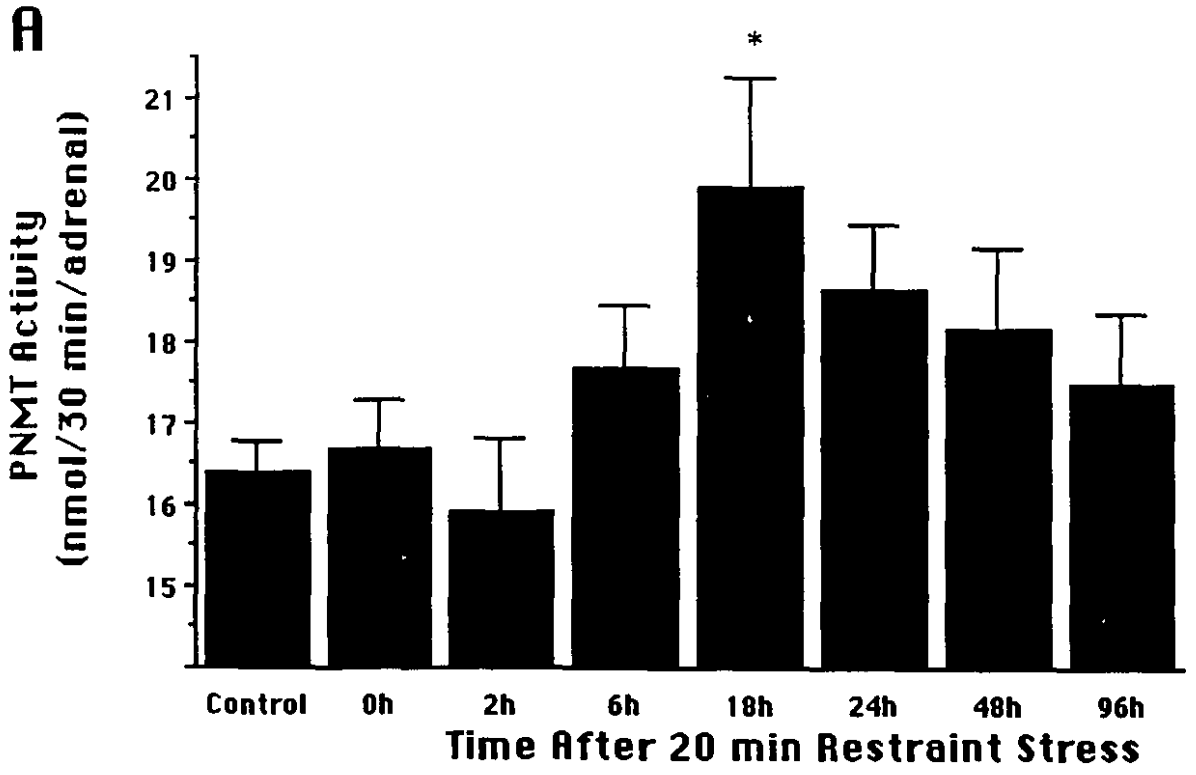
*CA content*

The regulation of CA biosynthesis by a single episode of acute stress generally is observed with a time delay of 18-36h in the different experiments in the present study. However, in the experiments using intact animals, adrenal DA was found to increase significantly immediately following the restraint stress (Figure V-2A). The increase in DA at the end of the 20 min restraint period may be the result of the trend observed in TH activity to increase immediately following the stressor (see Figure V-1B). In support of this, one study has shown an increase in the in vivo DA synthesis and rate of tyrosine hydroxylation during a 1h immobilization stress, whereas the increase in synthesis of NA and A are seen only 24h following the 1h stressor (Kvetnansky et al., 1971). Therefore, although increases in DA content can be observed immediately following restraint in our

studies, significant increases in A and NA are only observed at 18h, a time point that is consistent with the increases in enzyme activity.

This study and a few others (Kvetnansky et al., 1970; Kvetnansky, 1973; Hoffman et al., 1975; Bhagat and Horenstein, 1976) have provided evidence that prolonged stress is not required to regulate CA biosynthesis, as had been previously thought. We have shown that increases in the activity of both PNMT and TH occur following a mild stressor (20 min restraint) if measured with a time delay window of 18-36h. Similarly, longer more severe stressors (1h cold, 2.5h immobilization and 1-2h forced swimming), have been shown to increase TH and/or PNMT activity following a time delay of 6-32h. However, of importance is the time delay window used to measure the increase in CA biosynthetic enzymes, since this factor may markedly influence the interpretation of such studies (cf. our DEX suppression study). The present study supports our in vitro observations using cultures of bovine adrenal medullary cells where increases in PNMT activity have been observed in response to a short (2h) pulse of cortisol, with a time delay as short as 18h following the beginning of the pulse (Betito et al., 1992; 1993). These in vivo and in vitro studies suggest that GCs are important for the replenishment of CA stores lost during an acute stressor, by way of increase in CA biosynthetic enzymes. The time delay involved in this regulation suggests that at the level of the adrenal medulla, immediate changes in CA levels during the stressor is unneccessary, and that this increase will occur when needed in order to replace released CAs.

**Figure V-1. The effect of a single 20 minute restraint stress on the activities of adrenal PNMT and TH.** Rats underwent a 20 min period of restraint stress and were sacrificed by rapid decapitation either immediately following the end of the stressor (0h) or replaced into the home cage and sacrificed at various times (2, 6, 18, 24, 48, and 96h) following the beginning the stressor. Control animals were unstressed rats sacrificed without prior manipulation. Right adrenals were assayed for PNMT activity (A) and left adrenals for TH activity (B) as described in Experimental Procedures. Each bar represents the mean ± SEM from groups of 8 rats except for the control group (n=12). Significant differences were assessed using one way analysis of variance with post-hoc Tukey's test (*$p<.05$ vs non-stressed control group).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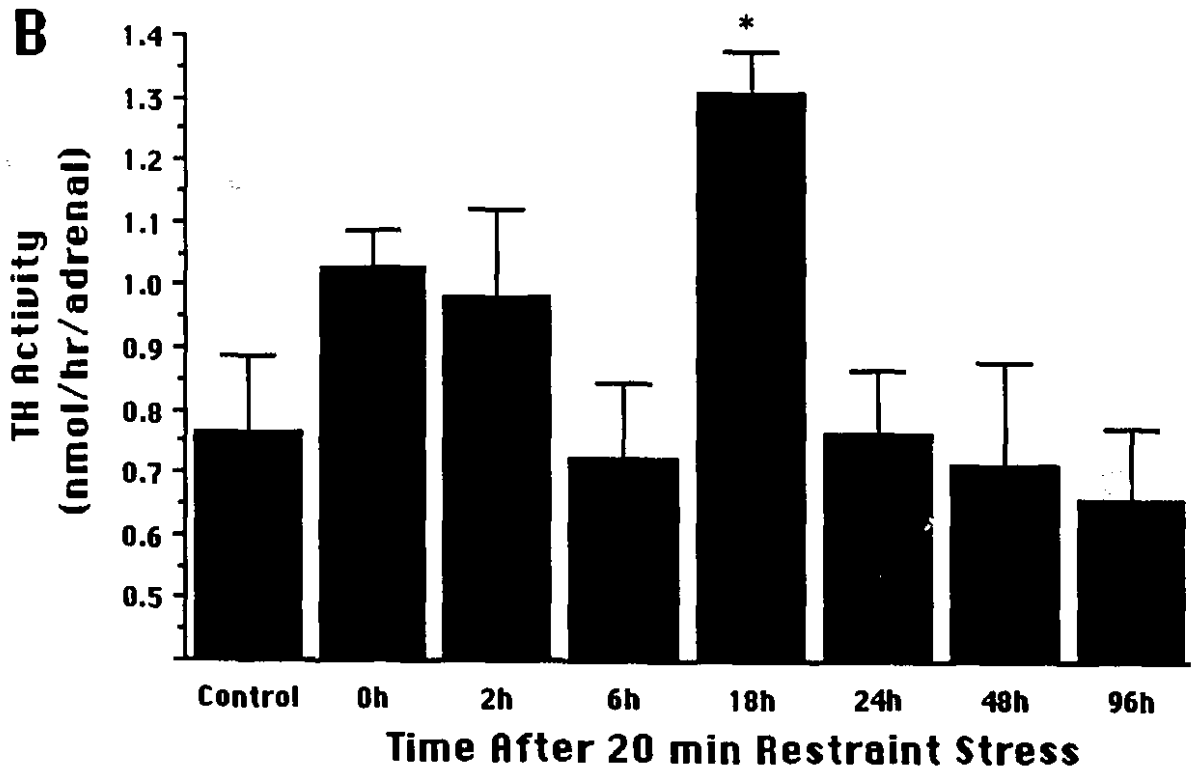
Time After 20 min Restraint Stress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TH Activity
(nmol/hr/adrenal)
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
*
Control
0h
2h
6h
18h
24h
48h
96h
Time After 20 min Restraint Stress

**Figure V-2. Adrenal CA content at various time points following a 20 min restraint stress.** The levels of dopamine (A), noradrenaline (B), and adrenaline (C) were measured using HPLC in homogenates of the right adrenals used for PNMT measurements in Figure V-1A. Each bar represents the mean ± SEM from groups of 7-8 rats except for the control group (n=12). (* $p<.05$ compared to the control group).

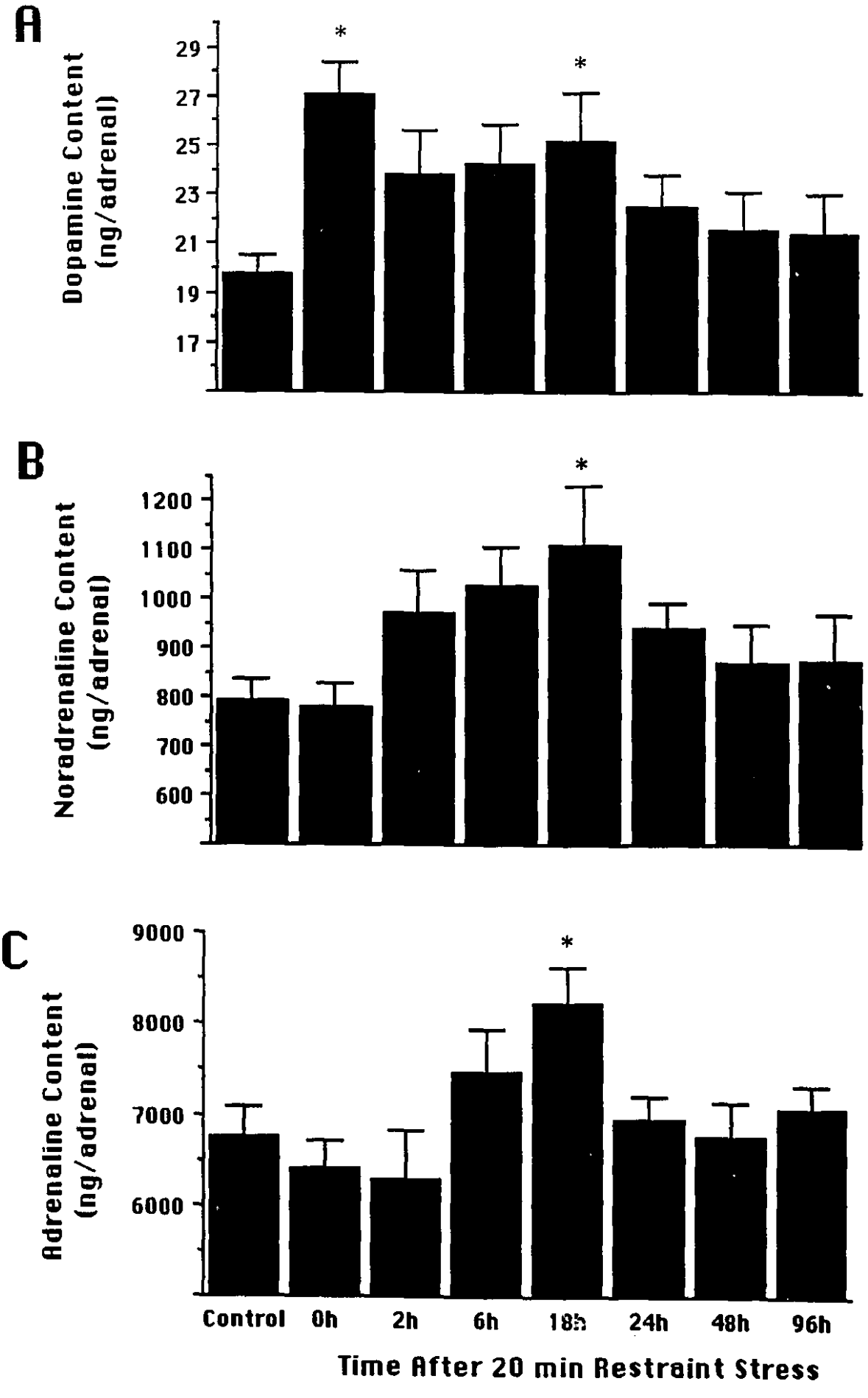

A
Dopamine Content
(ng/adrenal)
29
27
25
23
21
19
17
*
*
B
Noradrenaline Content
(ng/adrenal)
1200
1100
1000
900
800
700
600
*
C
Adrenaline Content
(ng/adrenal)
9000
8000
7000
6000
*
Control
0h
2h
6h
18h
24h
48h
96h
Time After 20 min Restraint Stress

**Figure V-3. The effect of DEX on the suppression of stress recovery plasma B concentrations.** Rats were injected with 100 μg/kg DEX subcutaneously 3h prior to a 20 min restraint stress. Tail vein blood samples were taken immediately before the stressor (pre sample) and at various time points following the stressor. Plasma B was measured using radioimmunoassay as described in Experimental Procedures. Each point represents the mean ± SEM from groups of 8 rats. Where no SEM is shown, the SEM is smaller than the plot symbol. (**$p<.001$ compared to vehicle treatment at same time point).

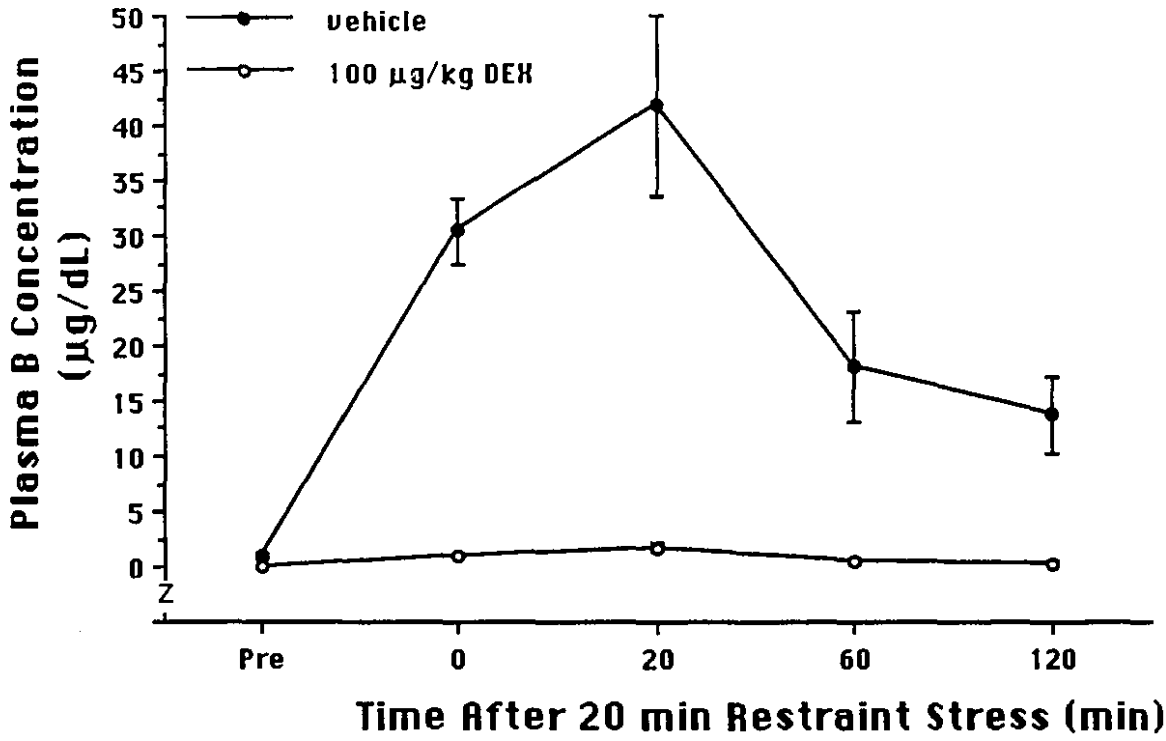
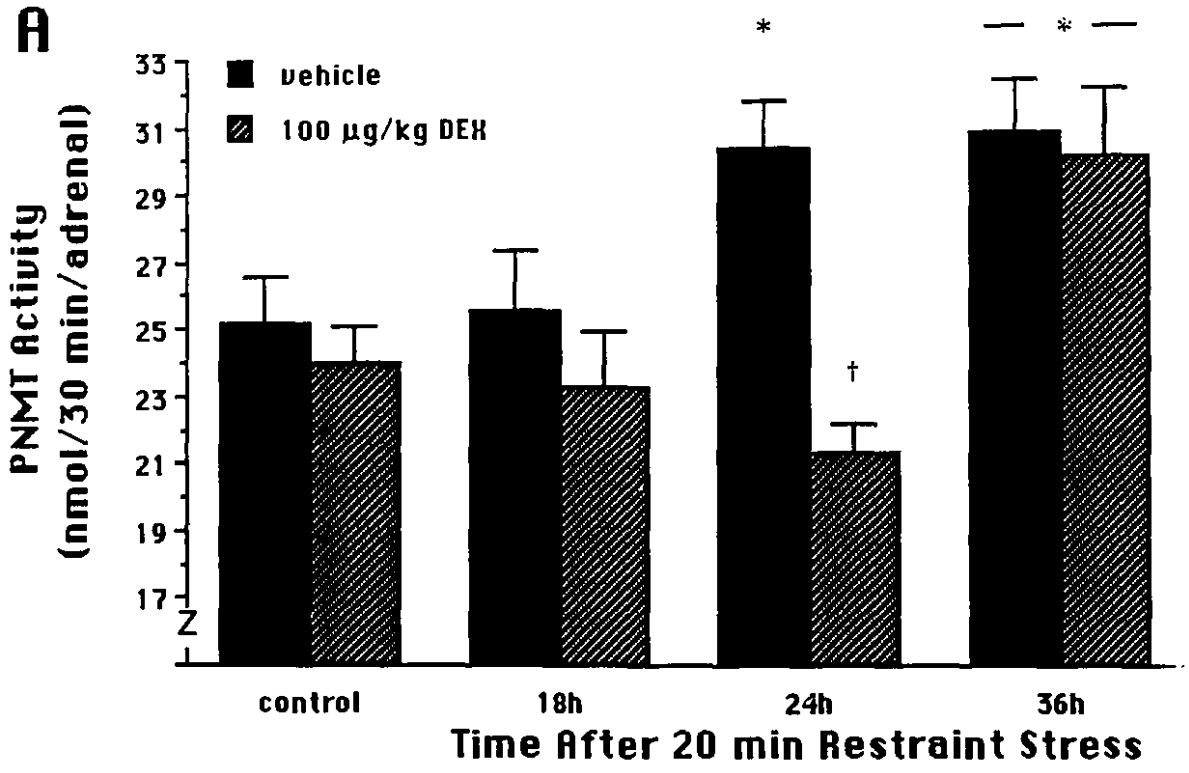

vehicle
100 µg/kg DEX
Plasma B Concentration (µg/dL)
50
45
40
35
30
25
20
15
10
5
0
Pre
0
20
60
120
Time After 20 min Restraint Stress (min)

**Figure V-4. The effect of DEX suppression of endogenous B secretion on acute stress-induced increases in PNMT and TH activities.** Three hours prior to the 20 min restraint stress, rats were injected with 100 μg/kg DEX in saline:ethanol (9:1). Control unstressed rats were injected with DEX 27h prior to sacrifice. Stressed rats were sacrificed at 18, 24 and 36h following the beginning of the stressor. Each bar represents the mean ± SEM from 7-8 rats for each time point for activities of PNMT (A) and TH (B). (Effect of DEX treatment: †$p<.001$ compared to vehicle treatment at same time point. Effect of time: *$p<.05$, **$p<.01$ compared to control unstressed rats for each condition).


A
vehicle
100 µg/kg DEX
PNMT Activity
(nmol/30 min/adrenal)
33
31
29
27
25
23
21
19
17
*
†
*
control
18h
24h
36h
Time After 20 min Restraint Stress

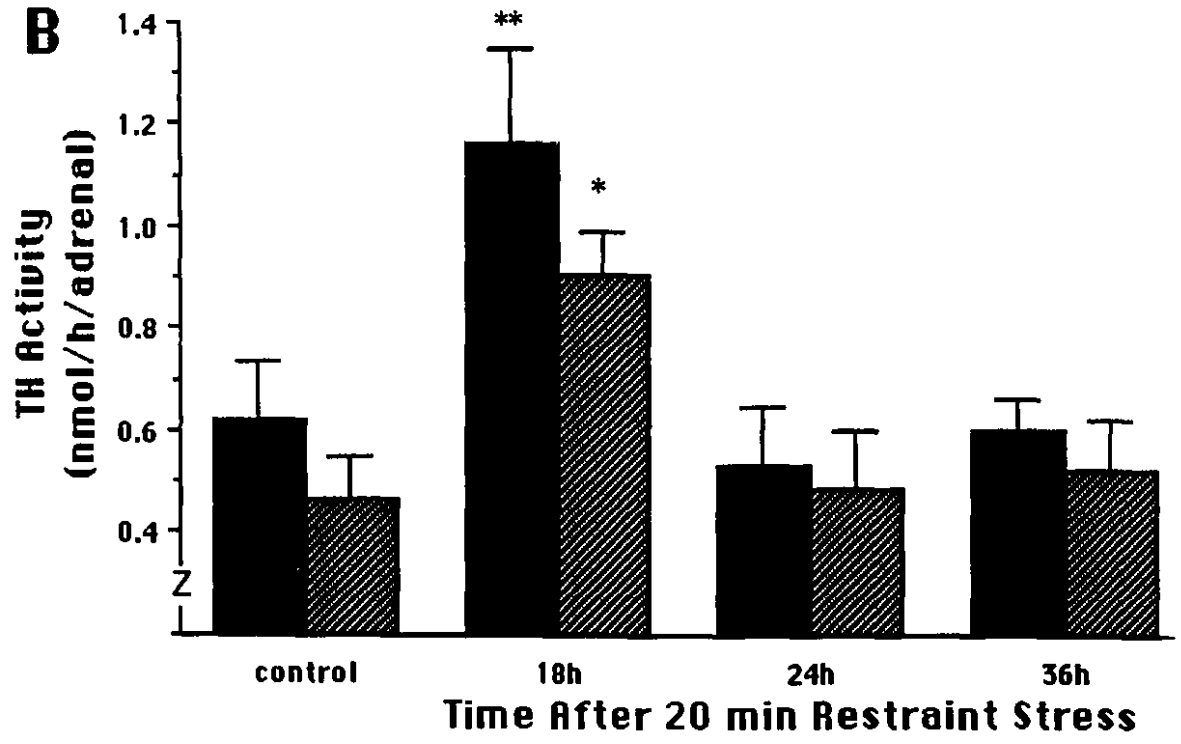

B
TH Activity
(nmol/h/adrenal)
1.4
1.2
1.0
0.8
0.6
0.4
**
*
control
18h
24h
36h
Time After 20 min Restraint Stress

**Table V-1. The effect of a single 20 min restraint stress on plasma ACTH and B at various time points following the stressor.**

| | ACTH (pg/ml) | Corticosterone (B) (μg/dl) |
|---|---|---|
| Control | 125.2±8.8 (12) | 1.41±0.40 (13) |
| 0 h | 1158.7±282.5 (8)* | 24.47±2.84 (7)* |
| 2h | 168.3±18.2 (8) | 8.92±2.85 (8)* |
| 6h | 139.3±22.3 (8) | 5.72±1.53 (8) |
| 18h | 172.2±25.1 (8) | 5.14±1.52 (8) |
| 24h | 118.7±19.9 (8) | 7.06±1.07 (8) |
| 48h | 76.1±9.5 (4) | 3.17±1.37 (6) |
| 96h | 123.0±32.0 (8) | 5.16±3.53 (8) |

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## Chapter VI. General Discussion

The present thesis has examined the adrenomedullary GC receptor, focussing on the characterization of the dynamics of this receptor, the regulation of levels of this receptor in response to a number of treatments, as well as an important functional consequence of GC receptor activation at the level of the adrenal medulla - the regulation of PNMT activity. The main impetus for studying the adrenomedullary GC receptor is the interaction between the adrenocortical GCs and the adrenomedullary CAs, an interaction due to the unique association of the adrenal cortex and the adrenal medulla, two tissues of different embryogenic origin. This anatomical association prompted us to carefully examine the adrenomedullary GC receptor dynamics for similarities to GC receptors in other tissues. We have shown that the pharmacology of the adrenomedullary GC receptor is novel compared to that of GC receptors found in other target cells. We show that the adrenomedullary GC receptor responds not only to low (nanomolar) GC concentrations, but also to high GC concentrations similar to those normally found bathing adrenomedullary cells. The adrenomedullary GC receptor can be regulated both by its own ligand, as in other tissues, and by cyclic nucleotide analogues. However, the direction of the changes in receptor levels in response to cAMP differs from those found for the GC receptor in other tissues and cells. Another difference is the ability of both cAMP and cGMP analogues to regulate GC receptor levels in the same direction, rather than in opposite directions as for other cell types. In addition, we provide novel evidence that GCs play a more important role in the dynamics of the regulation of CA biosynthesis than was previously thought. In essence, we show that a short-term increase in exposure of the adrenal medulla to GCs, whether in vitro or in vivo, can result in increases in PNMT activity if measured with a time delay. Therefore, although the finer points for the individual experiments have been discussed in the previous chapters, the following will elaborate some of those points and/or provide general interpretations on those findings.

## Characterization

The dynamics of the GC receptor have been well characterized in a number of tissues and cell lines. However, in adrenal medullary cells, little was known about this receptor other than its basic pharmacology (Kd and Bmax) and possible subtype. We are providing evidence for an in depth characterization of this receptor in bovine adrenal medullary cells in culture. The subtype of receptor has been confirmed using specific ligands for the GC receptor and competition studies. In addition, we have studied the dynamics of GC receptor translocation and nuclear uptake.

In order to characterize the adrenomedullary GC receptor, we have used radioligand binding techniques. This technique consists of incubating a soluble fraction prepared from cells, with radioligand for a period of 22h. Through this 22h "exchange assay", at which point endogenous cortisol bound to receptors will exchange with labelled GC (Kalimi and Hubbard, 1983), we are able to measure both hormone-bound GC receptors, and unbound non-transformed GC receptors. For the translocation study, this technique has allowed us to estimate the amount of receptors that translocate in response to a given concentration of cortisol by measuring a loss in soluble binding sites. Although this is an indirect measure of translocation of the GC receptor, it is supported by our studies which show uptake of radioligand into nuclear fractions of medullary cells. Unfortunately, for the GC receptor, a transformed receptor not bound to heat shock proteins cannot be measured by steroid receptor ligand binding (Mendel et al., 1986a; Sanchez, 1992). This therefore, precludes us from treating cells with steroid, preparing a nuclear fraction and estimating the levels of GC receptor bound to DNA by radioligand binding. In addition, these receptors cannot bind ligand if extracted off of the DNA by high salt (Scherrer et al., 1990) as can other steroid receptors. Therefore, we are limited in the technical ability to fully describe the dynamics of the GC receptor using ligand

binding techniques. Use of both ligand binding and immumocytochemistry concurrently would provide a more thorough characterization of steroid receptor dynamics.

The characterization of the dynamics of the GC receptor in the present thesis has been carried out over a wide range of cortisol concentrations. Nanomolar concentrations of cortisol were found to translocate the receptor following a 45 min pulse. Further translocation was also observed in response to higher concentrations of GCs more relevant to the adrenal medulla. These concentrations are in the range of $10^{-6}$ to $10^{-4}$ M, as measured in adrenal venous effluent in anesthetized (Kitay, 1961; Peytremann et al., 1973; Guidotti and Costa, 1974; Jones et al., 1977) and awake animals (Engeland et al., 1989). One might however argue that at the highest concentration of steroid used ($10^{-4}$ M), we are approaching the limit of solubility of the drug. Hydrocortisone used in the studies was in the form of the free base (MW=362.5 g/mole), which has a solubility of 15 mg/mL ($\approx 4 \times 10^{-2}$ M) in ethanol and 0.28 mg/mL ($7.7 \times 10^{-4}$ M) in water (Merck Index, 11th edition). Therefore, a $10^{-4}$ M solution of cortisol (hydrocortisone base), prepared with 1% ethanol in DMEM/F12 medium, should not reach the limit of solubility. Therefore, the highest concentration of cortisol used in the in vitro studies in the present thesis, since it is almost 10-fold lower than the reported solubility of this steroid in water, is most likely having an effect due to a real concentration and not a perceived concentration as may occur with other steroids.

In vivo reports of GC concentrations in adrenal venous effluent have been reported to be in the range of $10^{-6}$ to $10^{-4}$ M, and are measurements of total steroid levels. However, it is the free (non protein-bound) proportion of GCs which is physiologically active, making up approximately 10% of the total concentration (see Hammond, 1990). In vivo then, the medulla can be exposed to concentrations of free cortisol in the $10^{-5}$ M range. In vitro, we have observed a functional response at such high concentrations (increases in PNMT activity), following both continuous and pulse

exposure to GCs (see Chapters II and IV). A remarkable similarity can be observed in the translocation study and the PNMT activity study in Chapter II, where increased losses in soluble sites at the high GC concentrations are reflected by further increases in GC-induced PNMT activity. A regression analysis was performed to correlate the levels of soluble [$^3$H]-DEX binding with the increase in PNMT activity (data from Figures II-4B and II-6). This analysis revealed a significant correlation (F-test=277.5; P=0.001; $R^2$=0.986), suggesting that a loss in soluble sites in response to a particular GC concentration is reflected in an increase in PNMT activity. Therefore, we believe that it is valid to discuss GC receptor dynamics in terms of these higher concentrations.

However, even though there is a physiological correlate, it is possibile that the receptor dynamics observed at higher concentrations of cortisol ($10^{-6}$ to $10^{-4}$ M) may be a result of nonspecific effects of GCs. The point may then be raised that the high concentrations of GCs regulate PNMT by acting on the putative chromaffin cell membrane receptor for GCs (Inoue and Kuriyama, 1989). However, this seems unlikely since 1) the effect of the putative membrane receptor is rapid (order of seconds), whereas the effect of regulating PNMT requires a delay of at least 18h hours to be evident (see Chapter IV and Hersey and DiStefano, 1979; Kelner and Pollard, 1985), and 2) the activity of PNMT induced by both low ($10^{-7}$ M) and high ($10^{-5}$ M) concentrations of cortisol are most likely classical GC receptor-mediated since the GC receptor antagonist RU 38486 prevents this increase (Table II-1). These experiments taken together suggest that the effects of cortisol in concentrations as high as $10^{-4}$ M are not merely due to nonspecific membrane effects or activation of a putative membrane GC receptor.

An alternative mechanism to account for the dynamic response to high concentrations of cortisol could be the existence of an intracellular low affinity GC receptor, similar to the plasma membrane low affinity GC receptor of liver and CNS (Kd≈$10^{-7}$ M; Towle and Sze, 1983; Quelle et al., 1988; Orchinik et al., 1991). This

could account for the further increase in PNMT activity observed at high concentrations of GCs. However, this mechanism is unlikely to account for the loss in soluble sites (i.e. translocation) observed at high GC concentrations. The 22h exchange assay used to measure binding of [$^3$H]-DEX is performed at nanomolar levels of radioligand. Therefore, even if low affinity sites are translocating at the high concentrations of cortisol, it is the loss of high affinity sites which are being measured with nanomolar levels of radioligand. Control levels of GC receptors (i.e. prior to steroid treatment) in Figure II-4B, are levels of high affinity receptors since they are measured with nanomolar levels of radioligand. Therefore, any loss in soluble sites, which suggests translocation, are most likely lost high affinity sites.

In both the concentration-response curves for translocation and PNMT activity (in Chapter II), there are further significant increases in both parameters at $10^{-4}$ M compared to $10^{-5}$ M. Given that the adrenal medulla will be exposed to total concentrations in the $10^{-6}$ M to $10^{-5}$ M range basally, possibly rising to the $10^{-4}$ M range during stress in the awake animal, it would be physiologically important to be able to increase GC receptor actions (translocation followed by PNMT induction) to a further extent in response to stress. Paradoxically, with ligand binding studies, we show a saturation of the adrenomedullary GC receptor in the range of $10^{-8}$ M. This would suggest that no further translocation or functional effect could occur at concentrations of steroid above that level. Nevertheless, following GC treatment of <u>intact</u> cells, we do observe further increases in both translocation and PNMT activity at higher GC concentrations. At lower concentrations ($<10^{-6}$ M), we observe plateau changes in both parameters, with the further increases above this concentration suggesting that there is a cellular mechanism that allows for this increase. A possible mechanism for regulating the amount of steroid seen by the GC receptor is the presence of an intracellular corticosterone binding globulin (CBG) within chromaffin cells. CBG is a high affinity binding protein for GCs, found in

plasma ("CBG"), in rat pituitary and kidney cells, and bovine artery ("transcortin"; DeKloet and McEwen 1976; Feldman et al. 1973; Hayashi and Kornel 1990), and in adrenal cortex (Cochet et al. 1977; Saez et al. 1977). A similar protein may be present in adrenal medulla, and may saturate at a concentration in the $10^{-6}$ M range, leaving more free steroid available when total steroid concentrations exceed this range. This would then translate into further translocation and PNMT induction. Therefore, a likely possibility is that the adrenal medulla behaves like several other GC target tissues where the amount of steroid seen by the receptor is buffered by a binding protein (most likely intracellular), thus allowing higher concentrations of cortisol to produce additional functional effects.

The dynamics of the GC receptor as characterized in Chapter II showed a translocation of the receptor following a 45 minute exposure to nanomolar concentrations of cortisol (Figure II-4B). In Chapter III, we show that a much longer exposure (4 days) of cells to these same cortisol concentrations did not produce translocation of the GC receptor (Table III-4). Our inability to observe a translocation following 4d exposure to cortisol (given that the concentration was maintained over the 4d period) raised the possibility that adrenal medullary cells may initially respond to cortisol by translocating the GC receptor, but that prolonged exposure to cortisol does not result in continuous translocation of the GC receptor. However, this has not been tested in this or any other system and it remains unclear what the long-term GC receptor dynamics are. Intuitively, one might expect that once a gene is activated or repressed by GC actions, the need for continuous activation is unneccessary. In the adrenal medullary system, we have shown that regulation of PNMT does not require continuous exposure to cortisol in order to have an effect on induction of this enzyme (chapters II and IV). Therefore, this observation correlates with the lack of continuous translocation observed when cells are exposed to cortisol in concentrations in the nanomolar range for 4d.

The present thesis has therefore advanced our knowledge of the dynamics of the adrenomedullary GC receptor. We show that this receptor, like the GC receptor in other tissues, can respond to nanomolar levels of steroid. However, we show that the adrenomedullary GC receptor is unique. This receptor can respond, both on a dynamic and functional level, to much higher GC concentrations than what is found in the general circulation, levels much higher than the Kd for this receptor. For the concentrations of GCs tested ($10^{-9}$ M to $10^{-4}$ M), the dynamics of the receptor correlate with the GC-induced functional response (i.e. increases in PNMT activity). For GC receptors in other tissues, receptors normally exposed to nanomolar levels of GCs, high concentrations of steroid have not been tested for their ability to affect the dynamics or functional response of GC receptor binding. This has not been investigated most probably due to the unlikelihood that GC receptors in tissues other than the adrenal will ever encounter such steroid levels. However, there remains a possibility that receptor dynamics and functional effect apparent at high GC concentrations may not be unique to the adrenomedullary GC receptor.

## Regulation

Regulation of the adrenomedullary GC receptor by several factors has been studied in the present thesis given the possible functional implications on CA biosynthesis of altering GC receptor levels. We have shown a decrease in levels of GC receptors in response to both GCs and to cyclic nucleotide analogues. However, the chapter on regulation of the GC receptor has served to expose some of the limitations to the use of bovine adrenal medullary cells, isolated using Percoll density gradients, for these types of studies. Depending on the method of isolation, the purity of the cultures vary. In chapter II, cells used were prepared according to the method of Trifaró et al. (1978). Differential plating experiments where the proportion of non-chromaffin cells were counted at time of

plating, revealed that 12-15% of the plated cells in the less pure cultures were non-chromaffin. However, in the subsequent chapters, cells were isolated using the method of Livett (1984) with a measured proportion of non-chromaffin cells much smaller (2-8%; our unpublished observations). Even still, the cAMP treatment was able to increase cortisol production in both the less pure cultures and in the differentially plated chromaffin-enriched cell cultures. Since cortical cells must produce a very high concentration of cortisol in order to supply the general circulation (where it is diluted approximately 100-fold), a small proportion of contaminating cortical cells is sufficient to produce high nM levels of GC in response to cAMP (see Chapter III). This raises a limitation of using adrenal medullary cell cultures as tools to study the effect of cAMP on various medullary functions, since these effects may be mediated indirectly via cortisol production. In the case of GC receptor regulation, our studies clearly showed that cAMP-induced reductions in GC receptors was not mediated indirectly via cortisol.

The ability of cyclic nucleotides to regulate levels of the GC receptor has been demonstrated in a number of GC target tissues. The adrenal medulla differs in this respect to most other tissues (other than the AtT-20 cell line) where cAMP increases levels of GC receptors. The decrease in GC receptors in response to cGMP observed in adrenal medulla has also been observed in fibroblasts (Oikarinien et al., 1984). Therefore, although the direction of the changes in receptor levels is not always the same depending on the GC target cell, the present studies support a general regulation of the GC receptor by cyclic nucleotide second messengers. A decrease in receptor levels in other cell types has been shown to correlate with a decrease in the biological response of the cell to GCs. This phenomenon has also been observed in cyclic nucleotide-treated medullary cells. We have shown that cells with lower levels of GC receptors (due to cAMP or cGMP analogue exposure) have a reduced ability to respond to GCs by increasing PNMT to a similar extent as vehicle-treated cells. Finally, adrenal medullary cells behave in a similar manner

to other GC target cells, with a decrease in GC receptor levels in response to extended GC exposure. Therefore, even though the adrenal medulla is exposed to exceptionally high GC levels is situ, the adrenomedullary GC receptor appears to have similar characteristics and regulation as GC receptors in other cell types, with a similar functional correlate observed following a manipulation of receptor levels.

The physiological significance of cyclic nucleotide- and GC-induced regulation of GC receptors in the adrenal medulla is as yet unknown. In vivo, the adrenal medulla is exposed to neurotransmitters or neuromodulators, some of which activate cAMP and others cGMP. Decreasing medullary GC receptor levels in response to long-term neural and hormonal input could be the organism's way of safeguarding against possible long term damage due to chronic stress. Under conditions of chronic intermittent stressors, adaptation to these intermittent stressors occurs with respect to GCs as well as CAs released into plasma. That is, less A and GCs are released with each subsequent stressor, with a faster and greater decrement in the plasma levels of these hormones compared to rats stressed for the first time. This has been shown to occur with stressors such as restraint or footshock stress (Konarska et al., 1989) or with 15 min handling, novelty or water immersion stresses (De Boer et al., 1990) with interstressor intervals of 24h. Therefore, a chronic release of GCs and activation of cyclic nucleotides may result in subsequent decreases in GC receptor levels, which in turn would reduce the GC-induced increases in PNMT activity, providing less CAs for release following stimulation. We can speculate that decreasing GC receptor levels in the adrenal medulla could serve as a feedback mechanism to turn off elevated production of A. It should however be emphasized that our in vitro model differs in many ways from the in vivo situation; importantly, we are exposing the cells chronically to the drug treatment, therefore mimicking what would be a continuous chronic stressor rather than an intermittent one.

Nevertheless, hormonal- or cyclic nucleotide-induced decreases in adrenomedullary GC receptor levels may serve a physiological role under conditions of repeated stress.

**Function**

The function of GCs as regulators of the activity of adrenomedullary PNMT has been known for almost three decades. However, in depth characterization of the nature and timing of this regulation was not previously available, and is now provided in the present thesis. An important finding of the present thesis is the ability of high concentrations of GCs to influence both receptor translocation and PNMT activity. As discusssed above and in the previous chapters, the total (free plus bound) concentration of cortisol reaching the adrenal medulla is in the range of $10^{-6}$ to $10^{-4}$ M. These concentrations are sufficient to induce PNMT activity both following continuous exposure (Chapter II) and 2-3d following a pulse of cortisol (Chapters II and IV). In Chapter II, the cells were maintained in a medium devoid of steroid or serum. Therefore, the increase in PNMT activity 3 days following a pulse of GCs of $10^{-5}$ and $10^{-4}$ M (free concentrations) may have been because the cells were more sensitive (than cells maintained in the presence of a basal or background level of steroid) to PNMT induction due to a chronic lack of GC exposure. However, this is unlikely since in cells pretreated with cortisol in a concentration expected basally ($10^{-6}$ M total), the increase in PNMT activity in response to a cortisol pulse ($10^{-4}$ M total) was found to be similar to the increase found in vehicle-pretreated cells (Figure IV-4, Chapter IV). Therefore, adrenal medullary cells do respond to pulses of GCs in a high concentration range by increasing PNMT activity several days later, in cells maintained either in steroid-free or steroid-containing medium.

In cells maintained in serum-containing medium, in which low nanomolar levels of GCs are found, pulses of high concentrations of GCs can also increase PNMT. In

Chapter IV, where the medium contained 10% fetal calf serum, a total concentration of $10^{-4}$ M cortisol added to the medium estimates a free concentration in the range of $10^{-5}$ M. The concentrations of cortisol, in pulse form, that increased PNMT activity were in the range of concentrations expected to be seen by the adrenal medulla ($10^{-6}$ M to $10^{-4}$ M total), with no increases found in response to lower pulse concentrations. This suggested that the high concentrations of cortisol were physiologically relevant in the regulation of medullary PNMT.

One of the major findings of the present work is the observed increase in medullary PNMT activity in response to short-term cortisol or nicotine pulse exposure. This finding has provided us with a technical tool to study the functional status of medullary cells in which the levels of the GC receptor had been manipulated by various agents. Previously, it was thought that long-term exposure of cells was required to regulate PNMT, both for GCs (Hersey and DiStefano, 1979; Kelner and Pollard, 1985) and for nicotine (Evinger et al., 1988; Stachowiak et al., 1990). This has precluded undertaking studies in adrenal medullary cells which correlate GC receptor levels with the functional endpoint of PNMT activity. That is, it was previously technically tricky to alter receptor levels and then expose the cells to long-term drug treatment to observe changes in PNMT activity, since it was unknown whether the changes in receptor levels would be maintained over the incubation time required to alter enzyme activity. However, we have shown that this is now possible for the GC receptor, due to the finding that PNMT activity can be regulated, after a lag period, by a short pulse of GCs or nicotine. This allows us to measure changes in enzyme activity at a particular point in time, with a given level of receptors, without worrying that receptor levels will change over this incubation time. Using this tool, we have shown that decreasing levels of receptor with cyclic nucleotides results in a compromised ability of the cells to respond to a cortisol pulse. This is consistent with findings in many cell types, where the levels of GC receptor

determine the magnitude of the functional response to GCs (Bourgeois and Newby, 1979; Vanderbilt et al., 1987; Distelhorst, 1989; Dong et al., 1990; Tanaka et al., 1992). Therefore, this will allow an undertaking of a number of studies in adrenal medullary cells that would have otherwise been unfeasible, allowing a further in depth look into the dynamics of the GC-PNMT interaction.

Most of the present work has concentrated on the regulation of PNMT by GCs using adrenal medullary cells as an in vitro model of endocrine regulation of the adrenal medulla. However, as mentioned in chapter IV, PNMT can be regulated by a number of neurotransmitters or neurohormones released by the splanchnic nerve or found in the bloodstream. Short-term cholinergic nicotinic receptor activation appears to be one other mechanism by which regulation of enzyme activity occurs (Figure IV-5), supporting the relevance of neuronal influence over this enzyme. In medullary cell cultures, there is a chronic absence of cholinergic stimulation. This may create an upregulation of the nicotinic cholinergic receptor, resulting in "denervation supersensitivity" as has been found for muscle ACh following denervation of the motor end plate (Fambrough, 1979; Gardner and Fambrough, 1979) and as has been suggested for the adrenal pheochromocytoma cell line PC12 (Robinson and McGee, 1985). Therefore, adrenal medullary cells maintained in culture may have upregulated nicotinic receptors, increasing the sensitivity of the cells to nicotine, which in turn may influence the time course and/or nature of the regulation of PNMT activity by nicotinic agonists. This point must be kept in mind when comparing in vitro findings with those observed in vivo.

In vitro, nicotine pulses in combination with GC pulses (roughly analogous to the intact situation in vivo) produce greater increases in PNMT activity (Chapter IV) compared to either treatment alone. This suggests that nicotine (or neural stimulation) may be acting via a different mechanism than GCs to regulate PNMT activity. Indeed, it has been suggested that the splanchnic nerve induces de novo synthesis of PNMT while

GCs both inhibit degradation of this enzyme (Ciaranello et al., 1975; Ciaranello, 1980) as well as increase its rate of synthesis (Wurtman and Axelrod, 1966; Ciaranello, 1978), and may therefore account for the synergistic effect of GCs and nicotine on PNMT activity in medullary cells in culture.

However, combination pulses of GCs and nicotine also produce earlier increases in PNMT activity (at 12h rather that at 18h for either treatment alone) than do either agent alone. Nicotine (or neural input) may therefore modulate the GC-induced regulation of this enzyme, at least in vitro. This notion is supported by an in vivo study recently performed in pigeons (Mahata and Ghosh, 1991). The authors showed that 7 day GC treatment increased A synthesis in innervated adrenals. However, in denervated pigeon adrenals, the GC-induced synthesis of A was more pronounced compared to innervated adrenals. This suggested that the splanchnic nerve partially prevents, and therefore regulates, GC-induced changes in A synthesis. However, this study showed opposite effects to what we have observed in bovine adrenal medullary cells in culture, where nicotine positively modulates the GC-induced increases in the activity of PNMT. Nevertheless, such studies support an interaction between both neural and hormonal inputs on the regulation of PNMT activity.

However, in the present thesis, our in vivo studies have shown that both TH and PNMT can be increased following a mild, acute stressor in the absence of endogenous B release, suggesting that neural influence is sufficient to regulate both PNMT and TH. Regulation of TH and PNMT by neural or hormonal influence seems to differ from strain to strain. Most studies use Sprague-Dawley rats whereas we have used Long Evans rats, a strain that we routinely use for stress studies in our laboratory. In mice and rats, strain differences have been found in the predominance of neural and hormonal influences on the regulation of CA enzymes (Ciaranello et al., 1972; Cooper and Stolk, 1979). For example, in the mouse DBA/2J strain, PNMT activity is regulated by neural influences in

addition to hormonal influences, whereas in both C57BL/Ka and CBA/J strains PNMT activity is not neuronally controlled (Ciaranello et al., 1972). In the Lewis (LEW) strain of rats, the regulation of both TH and PNMT can occur independently of adrenal innervation (Cooper and Stolk, 1979). In hypophysectomized LEW rats, both enzymes do not increase in response to chronic (4d) immobilization, suggesting dependence on GC release for regulation. Conversly, in the Fisher (F344) strain hypophysectomy does not interfere with the increases in TH and PNMT whereas denervation abolishes these increases, suggesting an enzyme regulation independent of pituitary influence and dependent on neural influence. Therefore, in various strains, the predominance of hormonal or neural influence over the regulation of CA biosynthetic enzymes may vary widely. In the Long Evans strain, in the present thesis, it seems that at least following a mild acute stressor of short duration such as 20 min restraint, increases in both PNMT and TH can occur independently of hormonal influence.

Although both neural and hormonal inputs can regulate PNMT activity, the relative importance of either influence may affect the time course of elevation of this enzyme. As discussed above, this is suggested by our in vitro studies with nicotine and cortisol. In our in vivo studies, the absence of GCs also time shifts the stress-induced increase in PNMT activity (see Figure V-4). Therefore, one of the important factors to examine in the hormonal regulation of PNMT activity is the time course requirements necessary to observe an increase in PNMT activity following short-term exposure to either drug treatment or a mild stressor. In our in vitro studies, to more closely dissect out the temporal dynamics of GC regulation of PNMT activity, we have used a 2h pulse of cortisol, a length of time that approximates the in vivo elevation in GC levels following a single episode of acute stress. A number of studies using various stressors such as hemorrhage (Cameron et al., 1986; Engeland et al., 1989), motion stimulus (Brodish and Odio, 1989) and restraint stress (Meaney et al., 1989; the present thesis), have shown

elevations in plasma GCs which last between 45 min and 3h following termination of the stressor. Therefore, the 2h pulse of GCs used in the in vitro system can be compared to the elevation in plasma GC levels observed in in vivo experiments.

The time course of increases in enzyme activity in the in vivo studies in the present thesis generally are reflected by concomitant increases in CA content in adrenals, with the exception of dopamine content as described in Chapter V. A regression analysis of our data gathered on the time course of regulation of PNMT activity and A content (from Figures V-1A, and V-2C) revealed a significant correlation between these two parameters (F-test=11.21; $R^2$=0.692; P=0.0204). However, an important question to consider is whether changes in enzyme activity are always reflected by changes in CA content. Upon an examination of a number of studies which have measured both PNMT activity and A content following several manipulations (eg. chronic or acute stress, hypophysectomy, or drug treatments), the regression analysis shown in Figure VI-1 was obtained. This analysis revealed a significant correlation between PNMT activity and adrenal A content, suggesting that a change in PNMT activity can be reflected by a concomitant change in A content following a number of different experimental manipulations.

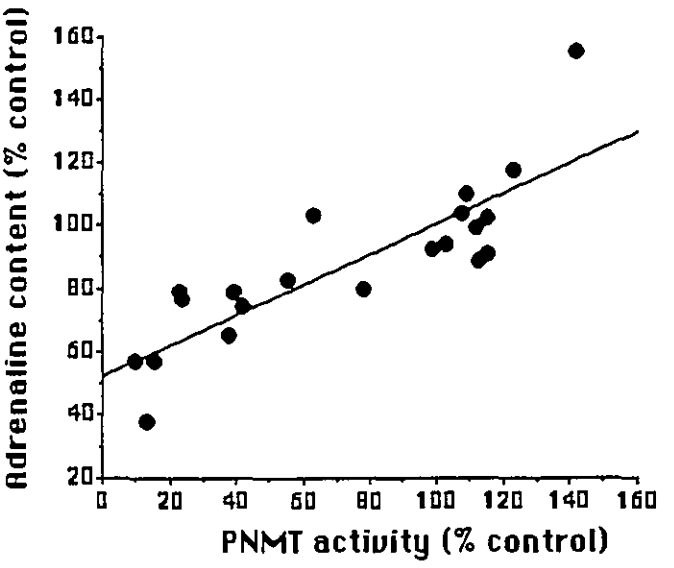


**Figure VI-1. Regression analysis of PNMT activity versus adrenal A content.** The data points were taken from studies which have measured both PNMT activity and A content following a number of manipulations such as hypophysectomy (Wurtman and Axelrod, 1965; 1966; Wurtman, 1966), steroid treatment (Wurtman et al., 1967), chronic stress (Kvetnansky et al., 1970) and acute stress (present thesis, Figures V-1A and V-2C). Analysis of Variance for regression on PNMT activity versus A content: F-test = 48.54; $p = 0.0001$; $R^2 = 0.719$.

Therefore, changes in PNMT activity are reflected in changes in adrenomedullary A content under a number of experimental conditions. In the present thesis, our in vivo study showed significant increases in A and NA above control values at 18h following a single episode of restraint stress (see Figure V-2). This increase parallels the increases in activity of both TH and PNMT. We have suggested that GCs increase PNMT activity, and consequently A content, in order to play a role in the recovery of the organism from a stressor. That is, the adrenal medulla has been able to replenish any CA stores that have been lost during the stressor. However, the adrenal doesn't seem to just replenish lost stores, but actually elevates CA content above pre-stress values. It is tempting to speculate that this overproduction of CAs is an adaptive response of the organism in anticipation of a second stressor. Unfortunately, very few studies have characterized CA metabolism during multiple periods of stress (i.e. stress-restress). Indeed, as mentioned

previously, during intermittent chronic stressors, less CAs are released with each subsequent stressor. This was demonstrated in one published paper where the response of the pituitary adrenal axis and the sympathoadrenal system to a second stressor is dependent on the interstressor interval (De Boer et al., 1990). With an interstressor interval of 24h, stressors such as 15 min handling, novelty or water immersion produce decreased release of A and GCs with each subsequent stressor. However, with interstressor intervals of 72h, responses to subsequent stressors are of equal magnitude to that of the first stressor. Therefore, this study seems to support the notion that replenishment of released CA stores occurs following a specific time delay between stressors. This notion may also be reflected in observations in the present thesis, where time delays of between 18h and 36h following GC or nicotine exposure were required to observe increases in enzyme activity and CA biosynthesis.

In conclusion, the present thesis has advanced our knowledge of the interaction between adrenocortical GCs and adrenomedullary CA biosynthesis. We illustrate that the functional implications of a GC-PNMT interaction are more dynamic than was previously thought. In vitro findings demonstrate that short-term exposure of adrenomedullary cells to either GCs or nicotine can elevate the activity of PNMT, if this activity is measured with a time delay. These findings have been confirmed in vivo, where we show increases in PNMT, TH and CAs following a time delay, in response to a mild acute stressor. Therefore, the present thesis has used both in vitro and in vivo methodology to provide novel observations on the characterization, regulation and function of the adrenomedullary GC receptor.

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Dear Ms. Davies,

I am a graduate student in the Department of Pharmacology at McGill University, studying under the supervision of Drs. Patricia Boksa and Michael J. Meaney. I am preparing my doctoral thesis and as a part of my dissertation would like to include results which have been published in *Neuroscience*. More specifically, I would like to include the data in the manuscript entitled "Glucocorticoid Receptors in Bovine Adrenal Medullary Cells in Culture: Regulation by Cyclic Nucleotides." published in Volume 54 (1), pp.263-273, 1993. The authors of this publication are K. Betito, J. Diorio, and P. Boksa.

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I thank you for your attention in this matter, and would appreciate your response as soon as possible.

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Katia Betito

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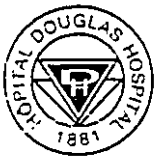


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Dear Mr. Hoekstra,

I am a graduate student in the Department of Pharmacology at McGill University, studying under the supervision of Drs. Patricia Boksa and Michael J. Meaney. I am preparing my doctoral thesis and as a part of my dissertation would like to include results which are to be published in *European Journal of Pharmacology*. More specifically, I would like to include the data in the manuscript entitled "Brief cortisol exposure elevates adrenal phenylethanolamine N-methyltransferase after a necessary lag period." which was accepted for publication in *European Journal of Pharmacology* on April 27, 1993 (Manuscript No. 707). The authors of this publication are K. Betito, J. Diorio, and P. Boksa.

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I thank you for your attention in this matter, and would appreciate your response as soon as possible.

Sincerely,

Katia Betito

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