PARENTAL CARE, ENVIRONMENTAL ENRICHMENT, AND HIPPOCAMPAL DEVELOPMENT AND FUNCTION IN THE ADULT RODENT

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ABSTRACT

This thesis examines the effect of early parental care and environmental experience on hippocampal development and cognitive function in the adult rodent. Maternal care in the rat contributes to cognitive function through effects on neural systems known to mediate certain forms of learning and memory. The offspring of mothers that exhibit a low frequency of licking/grooming (Low-LG mothers) over the first week of life show decreased hippocampal synaptogenesis and N-Methyl-D-aspartate (NMDA) receptor mRNA expression, and poor spatial learning; relative to the offspring of mothers that exhibit high levels of maternal care. The results of cross-fostering studies provide evidence for a direct relationship between maternal behaviour and hippocampal development in the offspring of Low-LG mothers (Liu et al., 1997; 2000).

In 1949, Hebb described how exposure to environmental enrichment throughout the peripubertal period enhances maze learning. Further, relative to their counterparts living under standard laboratory conditions, rats exposed to environmental enrichment show increased hippocampal synaptogenesis, increased expression of genes encoding the NMDA receptor complex, and enhanced neuronal proliferation and survival (Falkenberg et al., 1992; Saito et al., 1994; Kempermann et al., 1997; Nilsson et al., 1999; Ickes et al., 2001). Thus, it is clear that the hippocampal formation retains its characteristic plasticity across the lifespan. A fundamental question that emerges from these studies, and forms the basis for this thesis, is whether exposure to environmental enrichment during adolescence can

reverse the effects of reduced maternal care early in life. Further, are the effects of environmental enrichment on cognitive function associated with changes in the underlying neural mechanisms affected by maternal care?

Indeed, peripubertal environmental enrichment reversed the effect of reduced maternal care on hippocampal-dependent learning and memory, synaptic density, and several genes encoding for glutamate receptor subunits. For example, hippocampal NR2A, NR2B, GluR1 and GluR3 mRNA expression was reversed, with the strongest effects occurring in area CA1 of the hippocampal formation. Interestingly, the effect of enrichment on GluR1 and GluR3 mRNA expression was lateralized to the right hippocampus. Environmental enrichment did not reverse the effect of reduced maternal care on NMDA receptor binding or long-term potentiation in the dentate gyrus. In every instance where reversal occurred after exposure to enrichment during adolescence, the effect was specific to the offspring of Low-LG mothers, with little or no effect on the offspring of High-LG mothers. It is suggested that an increased level of astrocyte expression in the hippocampus of Low-LG offspring at the time of weaning could account for the individual differences in sensitivity to environmental enrichment. The findings are extended with the presentation of a new model where the issue of a paternal contribution towards offspring cognitive development is addressed in the monogamous California mouse (*Peromyscus californicus*).

Cette thèse étudie les effets des premiers soins parentaux et de l'expérience environnementale sur le développement de l'hippocampe ainsi que sur la fonction cognitive chez le rongeur adulte. Il est d'abord démontré que les soins maternels ont un impact sur la fonction cognitive du rat en affectant les systèmes neuraux

médiateurs de certaines formes d'apprentissage et de mémoire : les rejetons de mères démontrant une basse fréquence de stimulation tactile au cours de la première semaine de vie (les mères à LG faible) affichent une baisse de la synaptogenèse de l'hippocampe et de l'expression du mRNA du récepteur N-Methyl-D-aspartate (NMDA) et un apprentissage de l'espace lacunaire comparativement aux rejetons bénéficiant d'un niveau élevé de soins maternels. Ces résultats viennent appuyer des études s'intéressant à l'adoption chez le rat, qui révèlent l'existence d'une relation directe entre le comportement maternel et le développement de l'hippocampe des rejetons de mères à faible LG (Liu et al., 1997; 2000).

De plus, l'exposition à un environnement enrichi au cours de la puberté augmente l'apprentissage, comme l'a démontré Hebb en 1949. Les rats exposés à un environnement enrichi bénéficient d'une augmentation de la synaptogenèse de l'hippocampe par rapport aux rats vivant dans des conditions standard, d'une plus grande expression des gènes formant le complexe du récepteur NMDA et d'une hausse de la prolifération et de la survie des neurones (Falkenberg et al., 1992; Saito et al., 1994; Kempermann et al., 1997; Nilsson et al., 1999, Ickes et al., 1992). L'hippocampe conserve donc sa plasticité caractéristique à travers toute la vie du rongeur. Une question fondamentale qui émerge de ces études, et sur laquelle se base cette thèse, est à savoir si l'exposition à un environnement enrichi pendant l'adolescence peut renverser les conséquences de soins maternels faibles; les effets de cet enrichissement n'impliquent-ils pas certains changements dans les mécanismes neuraux affectés par les soins maternels?

En effet, l'enrichissement à la puberté renverse l'effet des soins maternels faibles sur l'apprentissage et la mémoire, sur la densité synaptique et sur plusieurs gènes qui encodent les sous-unités réceptrices de glutamate. Par exemple, l'expression mRNA des NR2A, NR2B, GluR1 et GluR3 de l'hippocampe est renversée (de façon plus visible dans la région CA1 de l'hippocampe), et l'effet de l'enrichissement sur l'expression de GluR1 et GluR3 mRNA se concentre dans le côté droit de l'hippocampe. Par contre, l'enrichissement n'annule pas les séquelles de soins maternels faibles sur la liaison des récepteurs NMDA et sur le LTP dans le gyrus dentate. Fait intéressant : l'enrichissement réussit davantage aux rejetons de mères à LG-faible, alors qu'il a eu peu ou pas d'impact sur les rejetons de mères à LG-élevé. Par ailleurs, il semble qu'un haut niveau de l'expression d'astrocyte dans l'hippocampe des rejetons à LG-faible au moment du sevrage puisse déterminer la sensibilité d'un rat à l'enrichissement environnemental. Enfin, ces découvertes permettent la création d'un nouveau modèle où l'effet de la contribution paternelle sur le développement cognitif des rejetons est appliqué à la souris californienne monogame (*Peromyscus californicus*).

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> "Two emotions must be unusually strong in the great scientific scholar; a devotion to truth and a passion for reputation. Timidity should be abandoned, and scientific work should be approached without reservation". - Santiago Ramón y Cajal (1897).

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Abbreviations

AMPA ..... alpha-amino-3-hydroxy-methyl-4-isoxazole propionic acid
Arc ..... activity regulated cytoskeletal-associated protein
BDNF ..... brain derived neurotrophic factor
BrdU- ..... bromo-2'-deoxyuridine
bFGF- ..... basic fibroblast growth factor
CA1 ..... ammon's cornu 1
DG- ..... dentate gyrus
GCL- ..... granule cell layer
GFAP- ..... glial fibrillary acidic protein
GluR1 ..... AMPA receptor subunit 1
GluR3 ..... AMPA receptor subunit 3
LG- ..... licking/grooming
LTP- ..... Long-term potentiation
NeuN- ..... neuronal nuclear marker
NGFI-A ..... nerve growth factor-induced gene-A
NMDA- ..... N-methyl-D-aspartate
NR2B ..... NMDA receptor subunit 2B
NR2A ..... NMDA receptor subunit 2A
MWM ..... morris water maze
PSA ..... population spike amplitude
SGZ- ..... subgranular zone
SYN ..... synaptophysin
TUC-4- ..... immediately post-mitotic neuronal marker

Original Scientific Contribution to Knowledge

1. Maternal care increases hippocampal neuronal survival in the offspring of High- compared with Low-LG mothers with no differences in cell proliferation or rate of neuronal maturation in the neonate. Such effects may contribute, in part, to the influence of early maternal care on the development of hippocampal synaptic density, and markers of synaptic communication previously demonstrated by Liu et al (2000).

2. Demonstrates the sustained plasticity within the hippocampus, and the capacity for the reversal of previously established 'deficits' in synaptic density, glutamate receptor expression, and cognitive performance through environmental enrichment at later stages of development.

2. Maternal care over the first week of life enhances the expression of NR2B expression in the CA1 region of the hippocampus, resulting in improved performance in the Morris water maze. The effect of environmental enrichment at later stages in development is to reverse the group differences inn NR2B expression, and thus eliminate the effect of reduced maternal care on hippocampal-dependent learning/memory.

3. An increased population of astrocytes in the offspring of Low-LG mothers at the time of weaning could provide a basis for a more substantial neurogenic and/or synaptogenic response to environmental enrichment during the postweaning period,

and thus provide a mechanism for increased sensitivity to environmental stimulation across the lifespan.

4. Variations in biparental care occur as a result of neonatal handling and these changes in parental care can contribute to offspring cognitive performance in monogamous California mouse (*Peromyscus californicus)*. The data suggest that environmental determinants modulate, in a gender specific manner, the effects of biparental care on offspring cognitive development.

CHAPTER 1: INTRODUCTION

1.1. OVERVIEW- As an organism interacts with its environment, for example, a rat discovers a supply of food some distance from its burrow, behaviourally relevant changes occur. Structural reorganization also occurs in different areas of the brain; for example, new neurons are generated, and new synapses are made in a brain structure called the hippocampus and, upon repeated visits to the food source, these synaptic contacts are strengthened. As the rat returns repeatedly to its new cache, experience-dependent learning has taken place and it no longer needs to search. The hippocampal formation plays a vital role in acquiring and retaining new information about the world and, across the lifespan, the hippocampus retains its capacity for experience-dependent change. Early life and adolescence are two periods when the hippocampus is particularly sensitive to environmental stimuli; thus, a main objective of this thesis is to explore the mechanisms of experience-dependent learning and hippocampal function with respect to environmental experience during critical periods of development. Further, why and how does experience affect the development of the hippocampal formation during early life and alter its sensitivity to environmental stimulation across the lifespan?

The mechanisms that mediate experience-dependent learning in the mammalian hippocampus include a constant birth of neurons (a concept called neurogenesis), and perhaps more importantly, dendritic remodeling and changes in synaptic communication, a phenomenon known as synaptic plasticity. Environmental effects on synaptic plasticity are mediated by intracellular and membrane-bound

synaptic mechanisms. These cellular mechanisms have effects on synaptic transmission, observable both *in vitro* and *in vivo,* through changes in long-term potentiation (LTP- section 1.3.3), a long-lasting increase in neuronal excitability and synaptic efficacy, thought to be a synaptic representation of learning and memory (Kato et al., 1999; Morris et al., 2003).

Hippocampal synaptic plasticity, essential for spatial learning and memory, is modulated by the synaptic vesicle glycoprotein, synaptophysin, and the action of the excitatory amino acid glutamate at post-synaptic N-Methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-methyl-4-isoxazole propionic acid (AMPA) receptors (Morris et al., 1982; Otto and Eichenbaum, 1992; Zamanillo et al., 1999; Wittenberg and Tsien, 2002; Schmitt et al., 2003). NMDARs are the most abundant glutamate receptors found in the hippocampus, and assume a predominant role in the intracellular signalling cascade fundamental for experience-dependent changes in synaptic plasticity (Carroll and Zukin, 2002). NMDARs are central for initiating adaptation to environmental cues, participate in mediating neural responses to stressful events, and are important for hippocampal-dependent learning and memory (Morris et al., 1986; Bolhuis and Reid., 1992; Shimizu et al., 2000). The specific timing of NMDAR functional maturity, and the expression of the molecular subunits that make up the NMDAR complex are essential for normal brain development. Altering the expression and activity of NMDAR during critical periods of hippocampal development may interrupt, or delay, important processes such as neuronal migration, synaptogenesis, and the refining of synaptic connectivity, thereby leading to cognitive deficits in adulthood.

1.2.1. ANATOMY OF THE HIPPOCAMPUS- The hippocampal formation is an anatomically complex bilateral brain structure, with areas including Ammon's horn (CA1, CA2, CA3), the dentate gyrus, the entorhinal cortex, and the subiculum (Figure 1) (Amaral and Witter, 1989; McEwen and Margarinos, 1997). The entorhinal cortex functions as a primary gateway into, and, together with the subiculum, it provides a major output of the hippocampal formation via the perforant path (Amaral and Witter, 1989). Ascending fibres from superficial layers of the entorhinal cortex form synapses with granule cells in the molecular layer of the dentate gyrus, and also form bilateral connections with pyramidal neurons in area CA1 (Cajal, 1901; Steward and Scoville, 1976; Amaral and Witter, 1989). The neurons of the dentate gyrus extend their axons along the mossy fibre pathway to pyramidal neurons in the stratum radiatum area CA3. These neurons extend (Schaffer collaterals), and form synapses with apical dendrites of pyramidal cells in the lacunosum-moleculaire of area CA1, thereby completing the tri-synaptic circuit (Amaral and Witter, 1989). Efferent projections from area CA1 to the olfactory bulb, lateral septal nucleus, nucleus accumbens, amygdala and hypothalamus serve to integrate the hippocampal formation into a complex network of cortical and subcortical structures involved in many aspects of learning and memory (Van Groen and Wyss, 1990).

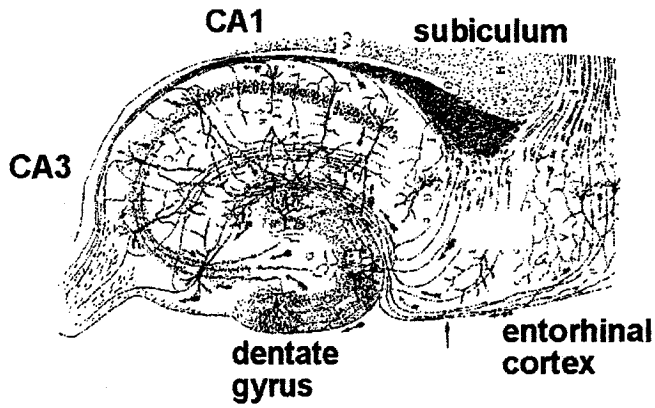


Figure 1. Representative schematic of the hippocampal formation (Cajal, 1901).

1.2.2. THE HIPPOCAMPUS, LEARNING AND MEMORY- The involvement of the hippocampal formation in different kinds of learning and memory is well established. Lesions of the hippocampus lead to impaired performance in a variety of spatial learning tests including the Morris water maze, radial arm maze, and in recognition learning tests such as the delayed matching to sample task (O'Keefe, 1993; Morris et al., 1982; Zola and Squire, 2001; Falkenberg et al., 1992; Kempermann et al., 1997; Kempermann and Gage, 1999; Rampon et al., 2000). Temporary inactivation of the dorsal hippocampus impairs acquisition of contextual fear conditioning (Matus-Amat et al., 2004). The data from these and many other studies has led to the suggestion that the hippocampus is critical, along with other systems, for processing information about contextual cues and the configuration and relevance of spatial cues in the environment (White and Macdonald, 2002; Lynch, 2004; Holscher, 2003). However, the hippocampal formation does not uniquely contribute to procedural learning or processing information about unconditioned aversive or fear provoking stimuli (DeCoteau and Kesner, 2000; Ledoux, 2000).

1.3. MECHANISMS OF SYNAPTIC PLASTICITY- During early development and throughout life, constant turnover of neurons in the dentate gyrus, along with the continuous reconfiguration of structural and functional synaptic connectivity in response to environmental cues, form the basis for the neural substrate of learning and memory. The following is a description of some of the factors thought to participate in structural organization of the hippocampus, synaptic plasticity, and hippocampal-dependent learning.

1.3.1. NEUROGENESIS- Beginning early in postnatal life and continuing across the lifespan, precursor cells continually proliferate, migrate, and differentiate into neurons in the subventricular zone, olfactory bulb and granule cell layer of the dentate gyrus (Altman and Das, 1965; Kempermann and Gage, 1999). While hippocampal neurogenesis persists across the lifespan of the rat, neuronal proliferation and survival is particularly intense during the early postnatal period (Bayer, 1982; Gould et al., 1997; Kudyrashov and Kudyrashov, 2001). Proliferation, differentiation, and survival of neurons in the hippocampus are supported by the expression of neurotrophic factors such as BDNF and βFGF, (McAllister et al., 1997; Yan et al., 1997; Wagner et al., 1999). Furthermore, new neurons have an increased rate of survival and enhanced function in response to environmental stimuli shortly after their birth, most notably, in response to experience-dependent learning (Shors et al., 2001; Gould et al., 1999).

1.3.2. EXCITATORY AMINO ACID RECEPTORS- During critical periods of development, the activation of excitatory amino acid receptors contributes to neuronal migration, synaptogenesis, and synaptic communication at the post-synaptic density (Collard et al., 1993; Yen et al., 1993; Behar et al, 1999). Post-synaptic glutamate receptors are expressed regionally throughout the hippocampus and belong to two distinct groups: 1) Ionotropic ligand-gated ion channels, comprised mainly of NMDA and AMPA receptors, and 2) G-protein coupled metabotropic receptors (mGluR). The literature regarding the entire glutamate receptor family is beyond the scope of this thesis, thus, the following review of the literature will focus on ionotropic glutamate receptors, with an emphasis on the NMDAR complex.

1.3.2.1. NMDA RECEPTOR- Across the lifespan, the expression of NMDARs and the trafficking of their internalized and membrane-bound subunits, are important dynamic processes that contribute to functional plasticity within the hippocampus (Barria and Malinow, 2002; Kitayama et al., 2003). Transient increases in NMDAR subunit mRNA and protein expression during the postnatal period directly effect neuronal migration, synaptogenesis, and refining of synaptic connectivity (Collard et al., 1993; Yen et al., 1993; Sheng et al., 1994; Wentzel et al., 1997; Behar et al., 1999; Sircar et al., 2000; Ritter et al., 2002; Pina-Crespo et al., 2002). NMDARs belong to the ionotropic family of excitatory amino acid receptors. They are glutamate-activated cation channels, characterized by a high Ca^{2+}/Na^{+} permeability ratio, a voltage-dependent Mg^{2+} block, and slow activation and deactivation kinetics

(Nowak et al, 1984; Ascher and Nowak, 1988; Johnson and Ascher, 1990; Schneggenberger et al., 1993; Monyer et al., 1994).

The hippocampal NMDAR complex consists of heteromeric assemblies of NR1, NR2A-D, and NR3A-B subunits. The ubiquitous NMDAR subunit NR1 combines with NR2B to form receptors with slower deactivation kinetics than those combined with NR2A (Fox et al., 1999). Slower deactivation of the NMDAR channel allows for greater Ca^{2+} influx and the potential for increased transcription of genes associated with enhanced synaptic efficacy (Fox et al., 1999). Conversely, incorporation of the NR3 subunit into the NR1/NR2 complex results in an NMDAR that is less sensitive to the excitatory action of glutamate, and shows a reduction in channel open time and conductance (Chatterton et al., 2002; Al-Hallaq et al., 2002). The role of the NR3 subunit in hippocampal-dependent learning and memory has not yet been determined.

There are two primary NMDAR heteromers expressed in hippocampal pyramidal and granule cells that contribute to the typical developmental profile of the NMDAR complex in the early postnatal life of the rodent. The immature form, with a high proportion of NR2B subunit and greater sensitivity to glutamate, is expressed as early as the first day of life (Monyer et al., 1994; Sircar et al., 2000). The mature form of the NMDA receptor complex, highest in NR2A, is less sensitive to glutamate, exhibits faster decay kinetics, and is increasingly expressed into adulthood (Fox et al., 1999; Sircar et al. 2000). The ratio of NR2A to NR2B subunit expression in the hippocampus increases across the postnatal period leading to a natural decline in

NMDAR sensitivity to glutamate-mediated excitation (Wenzel et al., 1997; Sheng et al., 1994; Ritter et al., 2002).

Decreased expression of NMDAR subunits and reduced sensitivity to glutamate leads to a decline in synaptic efficacy and spatial learning (Sakimura et al., 1995; Ito et al., 1997). Alternatively, increased expression of NMDAR subunits leads to an increase in NMDA-mediated sensitivity to glutamate, and enhances LTP and spatial learning; however, this also makes NMDAR-containing neurons more susceptible to the glutamate-induced excitotoxicity (Tang et al., 1999; Miyamoto et al., 2001). Thus, it is likely that active redistribution of NMDAR subunit expression occurring in response to environmental stimuli, contributes to synaptic plasticity in the hippocampal formation by increasing or decreasing the sensitivity of the NMDAR complex to the excitatory actions of glutamate.

1.3.2.2. AMPA RECEPTOR- AMPARs, also part of the ionotropic glutamate receptor family, are the principle mediators of fast excitatory neurotransmission in the hippocampus. Activation of co-localized AMPA receptors at the post-synaptic density facilitates the activation of slower acting NMDA receptors resulting in large increases in postsynaptic intracellular calcium, effects likely to be associated with learning-induced changes in neural plasticity (Malinow et al., 2000). A key mechanism in mediating these effects is the regulation of AMPAR receptor trafficking (insertion and internalization) of AMPARs at the post-synaptic density (Shi et al, 1999; 2001).

AMPARs are heteromeric complexes that are made up of 4 distinct subunits (GluR1-4) (Bettler and Mulle, 1995). There are two distinct subunit configurations of

the AMPAR complex in pyramidal neurons of the hippocampus; those containing GluR1 and GluR2, and those containing GluR2 and GluR3 (Wenthold et al., 1996). It is rare that AMPAR heteromers are comprised of the GluR1 and GluR3 combination. The GluR1/2 heteromeric complex is required for NMDA-dependent synaptic delivery of AMPARs to the PSD and the regulation of activity-dependent hippocampal synaptic plasticity. Thus, expression of GluR1 may be critical for changes in synaptic plasticity associated with hippocampal-dependent learning. The GluR2/3 complex is responsible for activity-independent recycling of AMPARs at the synapse, a process important for the maintenance of stable basal synaptic responses in hippocampal CA1 neurons (Shi et al., 2001, Passafaro et al., 2001; Meng et al., 2003; Andrasfalvy et al., 2003). GluR2/3 subunits are necessary, but not sufficient, for calcium permeability and the establishment of long-lasting functional circuitry within the hippocampal formation (Dong et al., 1997; Meng et al., 2003). Thus, the expression of GluR3 may be important for stabilization of newly formed synaptic contacts in response to learning.

Across critical periods of postnatal and juvenile development, the GluR1 subunit remains stable, with expression being the same at P35 as it is at birth (Ritter et al., 2002). On the other hand, the GluR2 and GluR3 subunits change their expression profile over time. The GluR2 subunit shows peak expression at P5-7 with a return to birth levels by P10, and a continuous decline to below birth levels by P18 (Ritter et al., 2002). Conversely, the GluR3 subunit begins very low at birth and shows a continuous increase until weaning (Ritter et al., 2002). Like the NMDAR complex, distinct changes in the pattern of AMPAR subunit expression across the

developmental period suggests that active redistribution of AMPAR subunit expression in response to environmental stimuli may be an important factor that contributes to sensitivity and stability of hippocampal synaptic networks, and thus contribute to synaptic plasticity of the hippocampal formation across the lifespan.

1.3.3. LONG-TERM POTENTIATION- The idea that plasticity of the synapse is a dynamic and continuous process in response to repeated stimulation was initially described by Donald O. Hebb in his book, *The Organization of Behavior* (Hebb, 1949). Hebb's postulate states that, "when an axon of cell A is near enough to excite a cell B and repeatedly and persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased" (Figure 2). In accordance with Hebb's postulate, it is now known that, across the lifespan, intense repetitive afferent stimulation can induce an enduring increase in synaptic transmission throughout the hippocampal formation (long-term potentiation, LTP) (Bliss and Lomo, 1973; Muller et al., 1988; Bekenstein and Lothman, 1991; Kramar and Lynch, 2003).

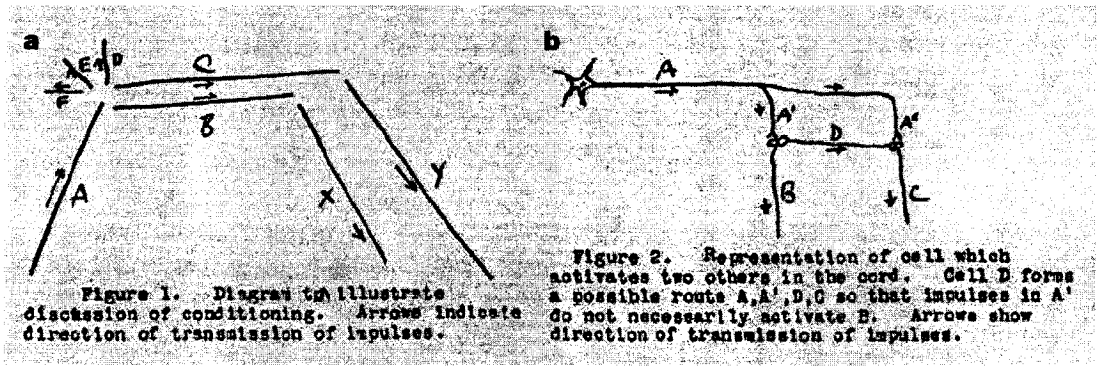


Figure 1. Diagram to illustrate discussion of conditioning. Arrows indicate direction of transmission of impulses.

Figure 2. Representation of cell which activates two others in the cord. Cell D forms a possible route A,A',D,C so that impulses in A' do not necessarily activate B. Arrows show direction of transmission of impulses.

Figure 2. Development of the "Hebb synapse" postulate (Brown and Milner, 2003).

In the hippocampus, postsynaptic NMDA receptors play an important role in the induction of LTP (Williams et al., 2003). Under basal conditions, the resting state of the ligand-gated and voltage dependent NMDA receptor is controlled by magnesium, blocking the influx of Ca^{2+} into the cell. When sufficient depolarisation of the neuron takes place, via the binding of glutamate to postsynaptic AMPA receptors, the magnesium block is alleviated and the NMDAR channel opens, thereby allowing Ca^{2+} to flow freely into the intracellular compartment. This influx of Ca^{2+} leads to a cascade of intracellular signal transduction events, sustained changes in activity-dependent gene expression, and a long lasting increase in synaptic efficacy (hence, NMDA-dependent LTP). Thus, the excitatory amino acid receptors, NMDA and AMPA, play an important synergistic role in the plasticity of synaptic communication in the hippocampus.

1.3.4. BASIC FIBROBLAST GROWTH FACTOR- Neurotrophic factors, originally identified as target-derived signals that regulate survival and differentiation of neurons, are now known to support neuronal excitability, synaptic development, and maintenance of synaptic plasticity (reviewed in Schinder and Poo 2000; Munno and Syed, 2003). βFGF or FGF-2 is a multifunctional growth factor found throughout the CNS, and is expressed at a particularly high concentration in the hippocampus during embryonic and postnatal development, and into adulthood (Gomez-Pinalla et al., 1994). Hippocampal βFGF participates in neuronal proliferation, differentiation, and survival, and its expression is protective for neurons born after exercise or learning (Ishihara et al., 1992; Gomez-Pinilla et al., 1998; Yoshimura et al., 2001).

1.3.5. SYNAPTIC PROTEINS- The postsynaptic density (PSD) serves as a general organizer of the postsynaptic signal transduction machinery, which links regulatory molecules to their targets, coordinates developmental and activity-dependent changes in postsynaptic structures, and establishes the functional topography of the postsynaptic membrane (Ziff, 1997). The PSD provides a structural matrix, and with the help of the cytoskeletal scaffolding protein, PSD-95, clusters ion channels in the postsynaptic membrane (Kennedy, 1997; Ehlers, 1999). Mechanisms involved in the regulation of synaptic efficacy, including long-term potentiation (LTP), are likely to have a basis in the PSD. For example, NMDA and AMPA receptors, both required for excitatory synaptic transmission, are clustered at the synapse via PSD-95 (Sheng and Kim, 1996; Wilson and Tonegawa, 1997; Schnell et al., 2002). Thus, PSD-95 contributes to critical features of synaptic integration and regulation.

Along with PSD-95, the synaptic vesicle glycoprotein, synaptophysin, is expressed in high concentration at the synapse. Synaptophysin is a specific marker of presynaptic terminals, and is often used as an index of synaptic density (Nakamura et al., 1999). Synaptic plasticity is associated with learning, and rats with spatial learning deficits have a significant reduction in synaptophysin expression (Van Reempts et al., 1992; Moser et al, 1994; Smith et al., 2000). Moreover, environmental enrichment increases hippocampal synaptic vesicle density, synaptophysin expression, and enhances hippocampal synaptic plasticity (Mollgaard et al., 1971; Saito et al., 1994; Nakamura et al., 1999). Indeed, recent reports demonstrate a direct relationship between increased hippocampal synaptogenesis and learning (Ramirez-Amaya et al., 2001; Leuner, Falduto and Shors, 2003; Leuner and Shors, 2004).

1.4. WHY FOCUS ON NMDARS? In contrast with the NMDAR complex, there is a relative paucity of knowledge concerning pharmacological and environmental influences on AMPARs, their subunit composition, and their role in hippocampal-dependent learning (for a review, see Riedel, Platt and Micheau, 2003). The reason for this is threefold; first, as of yet, agonists of AMPARs are very excitotoxic, making them difficult to use *in vivo*. Second, antagonists, even applied locally, prevent fast excitatory neurotransmission with essentially the same effect as a local anesthetic or GABA agonist. Finally, because of their intimate relationship with NMDAR function, AMPAR blockade leads to a disruption in NMDAR activity, thereby making it difficult to differentiate between the specific contribution of AMPA and NMDA receptor function for learning.

Recent gene targeting studies have begun to elucidate the role of AMPARs in hippocampal-dependent synaptic plasticity and learning. For example, GluR1 knockout mice have deficits in CA1 synaptic plasticity, a reduction in extra-synaptic hippocampal AMPA receptors, and show impaired spatial working memory (Zamanillo et al., 1999; Reisel et al., 2002; Schmitt et al., 2003; Andrasfalvy et al., 2003). However, since there are so few studies demonstrating a direct link between environmental experience, AMPAR function and hippocampal-dependent learning, and substantial data supporting the contribution of experience-dependent changes in NMDAR function and learning, the remaining overview of the literature will focus on how the environment can alter the structure and function of the NMDAR complex, and its role in hippocampal synaptic plasticity and learning.

1.4.1. PHARMCOLOGIC INFLUENCES- A number of studies have shown that stable NMDAR activity is essential for neural and behavioural development in both rodents and primates (Gorter et al., 1992a; Facchinetti et al., 1993; Gould and Cameron, 1997; Deutsch et al., 1998; Mohn et al., 1999; Popke et al., 2001; Popke et al., 2002). Blocking NMDARs during early postnatal development with the non-specific NMDAR antagonist MK-801, causes abnormal development of hippocampal synaptic circuitry in the rat, decreases patterned single-alternation discrimination learning in postnatal rats, and diminishes spatial learning with and without food reinforcement in early adulthood (Gorter and de Bruin, 1992b; Highfield, Nixon and Amsel, 1996; Luthi et al., 2001; Latysheva and Rayevsky, 2003). This effect is characterized by an abnormal increase in the frequency of spontaneous firing, a more complex dendritic arbor of CA1 pyramidal cells, and increased density of presynaptic boutons on CA3 afferent fibres (Luthi et al., 2001).

Chronic MK-801 treatment during early development also increases apoptosis in the neonatal hippocampus, and produces a long-lasting reduction in synaptic efficacy during the juvenile period (Ikonomidou et al. 1999; Bellinger et al. (2002). The effects of interfering with normal hippocampal NMDAR development are not limited to those induced by NMDA antagonists. Neonatal monosodium L-glutamate treatment (MSG-used to model increased glutamatergic activity resulting from over-activation of excitatory amino acid receptors during development) significantly increases NR1, 2A, and 2B mRNA expression, increases cell death, and adversely affects hippocampal are CA1 dendritic spine density in adulthood (Beas-Zarate et al., 2001, 2002). These neurochemical alterations have functional implications, as

animals treated with MSG are hyperactive and show learning impairments in adulthood (Ali et al., 2000). Taken together, these studies suggest that NMDAR activation during early postnatal development could determine the efficacy of synaptic remodelling process in adulthood.

The role of NMDAR activity in the refinement of neuronal networks during the postnatal period is not unique to the hippocampus, as there is substantial evidence to suggest a more ubiquitous role for NMDAR-mediated activity-dependent plasticity in the developing visual and somatosensory systems (Hubel and Wiesel, 1998; Fox et al., 1996; Yuste and Sur, 1999; Constantine-Paton, 1994). NMDAR antagonists applied to the superior colliculus during the first two postnatal weeks cause aberrant retinal axon arborization and a disruption in neural map formation (Simon et al., 1992). Further, barrel cortex development is disrupted by systemic and local application of NMDAR antagonists in neonatal rats, and transgenic mice carrying a deletion of the NR1 gene restricted to excitatory cortical neurons show atypical somatosensory cortical development (Mitrovic et al., 1996; Iwasato et al., 2000). In conjunction with the data on hippocampal development, these studies provide evidence that a disturbance in the normal developmental trajectory of the NMDAR complex can have long-lasting effects on structural reorganization and synaptic efficacy throughout the brain, and demonstrate the importance of the NMDAR complex for neural and behavioural development.

Experiments involving manipulation of the NMDAR complex in adulthood also provide evidence for the importance of stable NMDAR function for hippocampal-dependent learning. Blocking NMDAR activity in adulthood impairs

memory for socially-transmitted food preference, retention of habituation to novelty, acquisition of enhanced place preference conditioning, and spatial learning (Morris et al., 1986; Carey, Dai and Gui, 1998; Bevins and Bardo, 1999; Babcock et al., 2002; Roberts and Shapiro, 2002). In the adult rat, chronic MK-801 treatment disrupts hippocampal function by increasing NR1 mRNA expression in CA1, decreasing NR2A in CA3 and DG, while increasing NMDAR binding density throughout (Morris et al., 1982; Oh et al., 2001). These neurochemical changes associated with NMDAR blockade are most likely compensatory responses in gene expression that occur as a result of NMDAR inactivity that are a natural cellular response to the increased presence of glutamate at the synapse once the effects of the antagonist subside.

In some tasks, prior exposure to certain elements of the testing environment attenuates the effects of NMDAR antagonists. The effects of NMDAR blockade on hippocampal-dependent learning may ultimately depend on familiarity with environment. For example, although NMDA antagonists impair learning in the radial arm maze, Morris water maze, and contextual fear conditioning tasks (Kawabe et al., 1998; Alhander et al., 1999; Sanders and Fanselow, 2003), adult rats can perform successfully provided they are exposed to the strategies required to solve the task prior to drug administration (Shapiro and O'Connor, 1992; Saucier and Cain, 1995; Sanders and Fanselow, 2003). Interestingly, NMDA antagonists block the enhancing effects of novelty on retrieval of a previously learned task (Izquierdo et al., 2001). These studies emphasize the importance of stable NMDAR activity during initial responses to environmental cues, hence a role for the hippocampal NMDAR in

processes of adaptation, and their importance for hippocampal-dependent learning and memory.

The common theme emerging from these studies (and those to follow) is the requirement of a balancing act with regard to hippocampal NMDA subunit composition and NMDAR sensitivity to the excitatory activity of glutamate. Excessive expression, or too little for that matter, of any of the NMDA subunits leads to an imbalance in the stoichiometry of the NMDAR subunits, change in affinity for glutamate, and/or increased/decreased channel opening and Ca^{2+} influx. Subsequently, these abnormalities in function of the NMDAR complex could lead to unfavourable synaptic remodeling and decreased cognitive function, as well as an increase in susceptibility for hippocampus-related neurological disorders.

1.4.2. ENVIRONMENTAL INLFUENCES- Environmental factors that influence hippocampal NMDAR and cognitive development include those mediated by changes in parent-infant interactions during the early postnatal period. Maternal care has been shown to contribute to individual differences in cognitive development in humans and primates, as well as rodents (Ruddy and Bornstein, 1982; Bornstein, 1985; Tamaroff et al., 1986; Zaharia et al., 1996; Liu et al., 2000). In the rat, tactile stimulation derived from maternal licking/grooming (LG) of the young appears to be a critical factor for hippocampal development. The offspring of mothers that show increased pup licking/grooming exhibit increased synaptic density and NMDAR binding, elevated expression of the mRNAs encoding for the NR2A and NR2B subunits and

enhanced spatial and non-spatial learning, relative to the offspring of mothers that show decreased levels of maternal LG (Liu et al., 2000).

Studies investigating the effects of early-life experience such as neonatal handling and maternal deprivation on hippocampal development and cognition are starting to emerge. An important consideration to be made concerning these data is that the effects of early handling and maternal deprivation appear to be mediated, in part, by changes in parental behaviour towards the young (Levine, 1967; Smotherman, Brown and Levine, 1977; Villescas et al., 1977; Liu et al., 1997; D'Amato et al., 1998). Upon reunion, after a brief separation, mother rats tend to increase pup licking/grooming. Consequently, neonatal handling enhances cognition and diminishes age-related learning impairments (Meaney et al., 1988; Vallee et al., 1999; Tang, 2001). Neonatal handling increases hippocampal LTP, enhances synaptogenesis in the CA3 and dentate gyrus, and increases synaptic plasticity in CA1 (Wilson et al., 1986; Tang and Zou, 2002; Priebe, Giannotti, and Brake, 2002).

Maternal deprivation during the early postnatal period diminishes cognitive functioning and exacerbates age-related learning impairments (Oitzl et al., 2000; Huot et al, 2002; Garzon et al., 2002). Maternal separation during this critical period of hippocampal development decreases mossy fibre density, granule cell proliferation and increases apoptosis in the dentate gyrus (Lee et al., 2001; Huot et al., 2002.). Maternal separation also reduces hippocampal NR2A and NR2B mRNA expression in adulthood (Roceri et al., 2002). In the biparental rodent (*Octodon degus)*, short periods of biparental deprivation increase NMDAR binding density in CA1 and CA3, an effect that is blocked by presentation of maternal call during the separation period

(Ziabreva et al., 2000). It remains to be determined whether changes in hippocampal NMDAR binding density in *Octodon degus* are related to hippocampal-dependent learning. Taken together, these studies clearly suggest an influence of parental care on the efficient development and function of NMDAR and synaptic organization in the hippocampus across several species.

Exposure to environmental factors, such as lead (Pb), ethanol, and cocaine, during early postnatal development can alter hippocampal NMDAR subunit composition and impair hippocampal-dependent learning in adulthood (Olney et al., 2000; Nihei and Guilarte, 2001; Naassala and Daoust., 2002). For example, neonatal Pb exposure decreases hippocampal NR2A mRNA expression and increases NMDAR binding density in the neonate: effects that persist into adulthood and contribute to cognitive impairment (Rice, 1996; Nihei and Guilarte, 1999; Lasley, Green and Gilbert, 2001; Zhang et al., 2002).

Neonatal ethanol exposure increases mRNAs for the NR1, NR2A, and NR2B subunits, increases NMDAR binding density, but impairs spatial learning in rats (Naassala and Daoust, 2002; Johnson and Goodlett, 2002). Neonatal cocaine exposure increases in NMDAR binding density, and in adulthood, cocaine-induced seizures result in a transient up-regulation of NMDAR binding density in the hippocampus (Itzhak and Martin, 2000; Huber et al. 2001a, 2001b). These studies are largely correlational, thus, it has yet to be determined whether the observed increases in NMDA receptor binding and subunit expression that occur as a result of exposure to environmental toxins during the neonatal period actually have a direct impact on

synaptic communication in the hippocampus and with subsequent effects learning and memory in adulthood.

Across the lifespan, there is a natural decline in NMDAR binding density in CA1, CA3 and subicular regions of the hippocampus and a progressive decrease in spatial learning ability (Barnes, 1988; Wenk and Barnes, 2000). Furthermore, blocking hippocampal NR2B mRNA expression with antisense oligonucleotides mimics age-related impairments in spatial learning (Clayton et al, 2002). Dietary restriction increases hippocampal NR1 expression and changes the ratio of mRNA expression for NR1, NR2A, and NR2B in area CA1: in aged mice, a lower ratio of NR1 to NR2B mRNA is related to deficits in learning and memory (Magnusson, 2001). Thyroid hormone deprivation impairs learning and memory and decreases NR1 and NR2B mRNA expression (Fundaro, 1989; Lee, Brady, and Koenig, 2003). Conversely, growth hormone decreases NR1 mRNA, but increases NR2B mRNA expression (Le Greves et al., 2002).

Environmental enrichment during the peripubertal period increases hippocampal NR2A and NR2B protein expression, enhances neurogenesis in the dentate gyrus, and facilitates hippocampal-dependent learning (Kempermann et al., 1997; Nilsson et al., 1999; Rampon and Tsien, 2000; Tang et al., 2001). Interestingly, environmental enrichment reverses some of the effects of early life adversity on learning and memory in adulthood, and these effects appear to be mediated, in part, by a restoration of normal NMDAR subunit mRNA levels. For example, enrichment reverses the NR1 mRNA and spatial learning deficits in rats exposed to lead during the prenatal period (Guilarte et al., 2003). These studies further demonstrate the

ongoing adaptational capacity of the hippocampus, the role NMDARs play in supporting this plasticity, and their contribution towards cognitive function in adulthood.

1.5. STATEMENT OF THE ISSUE- Environmental experience during early life affects the structure and function of the NMDAR complex, and it is evident that these effects are not necessarily permanent. The question remains as to whether enrichment has specific effects during critical periods of development, or, if synaptic plasticity and the NMDAR complex are sensitive to environmental stimuli across the lifespan. Two factors based on the current literature allow us to directly address this; first, reduced maternal care early in life alters normal hippocampal development, and second, environmental enrichment during adolescence is enhances hippocampal development.

In the following series of experiments, we explore whether exposure to environmental enrichment during the adolescent period can reverse the effect of reduced maternal care early in life on cognitive function. We also examine how environmental enrichment can induce compensatory changes in the neural mechanisms underlying synaptic plasticity in hippocampal formation, and thereby "reverse" the effect of reduced maternal care on cognitive function in adulthood. In addition to the NMDAR complex, several other putative underlying neural targets associated with hippocampal synaptic plasticity (i.e. synaptophysin, AMPAR, LTP, the neurotrophic factor bFGF, GFAP expressing astrocytes, and neuronal survival) are explored. The findings are extended with the presentation of a model where the

issue of a paternal contribution towards offspring cognitive development is addressed. Finally, the significance of the data is discussed with respect to human intervention strategies and implications for cognitive function, and hippocampus-related disorders across the lifespan.

CHAPTER 2. EFFECT OF MATERNAL CARE ON NEURONAL SURVIVAL IN THE HIPPOCAMPUS OF THE RAT.

2. RATIONALE- With the use of an elegant cross-fostering paradigm, Liu et al (2000) clearly demonstrated the effect of maternal care on hippocampal-dependent spatial learning in the Long-Evans rat (Figure 3). These findings provide a basis for all of the experiments presented from this point onward.

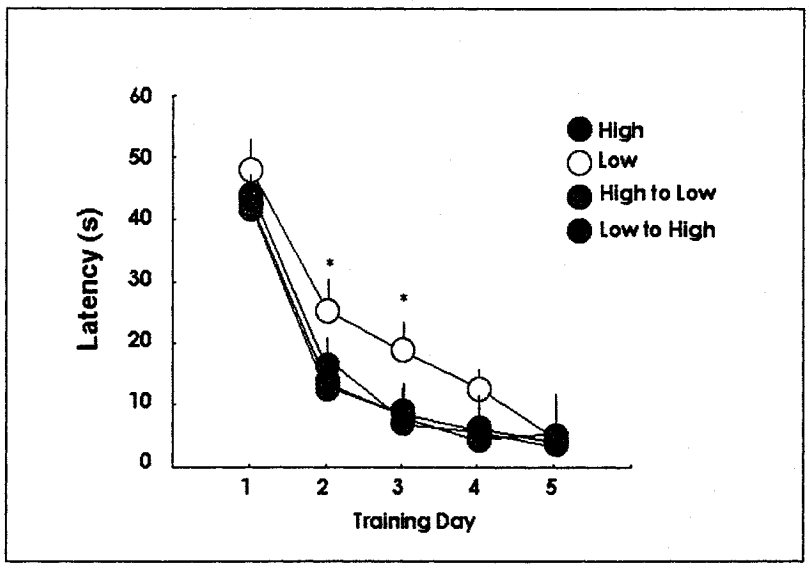


Figure 3. Mean ±SEM latency to find the platform in adult male offspring (n = 8/ group) born to Low-LG mothers and reared by either Low- or High-LG (low to high) mothers compared with the offspring of High-LG mothers reared by either High- or Low-LG (high to low) mothers (* $p < .05$). (from Liu et al., 2000).

Having established a functional link between maternal influence during the early postnatal period and cognitive function in adulthood, Liu et al. (2000) then set out to establish whether or not maternal care influenced the development of neural mechanisms known to contribute toward hippocampal plasticity and function.

Accordingly, maternal care affected both hippocampal synaptic density (Figure 4), and the expression of mRNA for the NMDAR subunits NR2A and NR2B in area CA1 and the DG of the hippocampal formation (Figure 5).

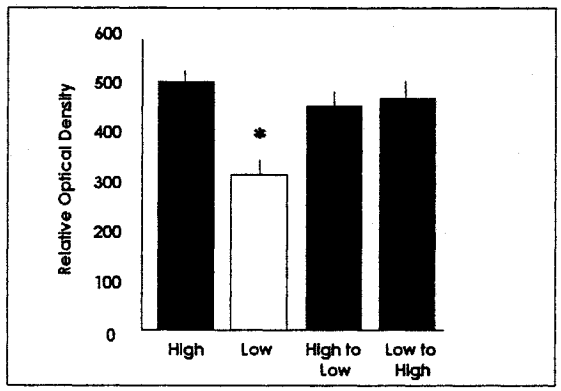


Figure 4. Mean ±SEM optical density units for synaptophysin protein expression from day 90 high-high, low-low, high-low, low-high animals (n = 6/ group drawn from 4 litters from each condition). Post-hoc analysis showed that synaptophysin immunoreactivity was significantly (* $p < .01$) lower for the low-low group by comparison with any other group (from Liu et al., 2000).

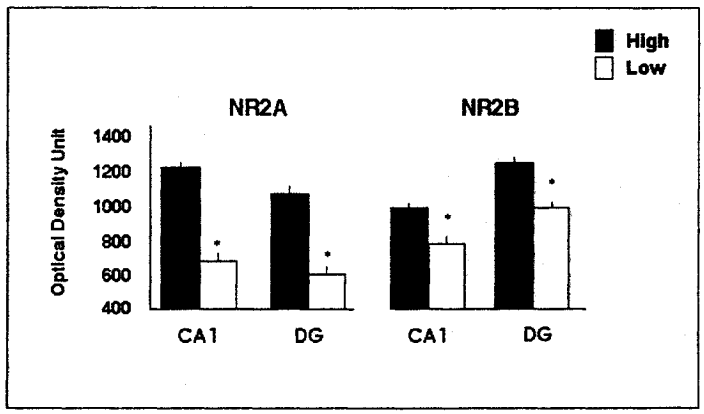


Figure 5. Mean ±SEM optical density units for NR2A and NR2B mRNA expression in the hippocampus of offspring of High- and Low-LG mothers (* $p < .05$) (from Liu et al., 2000).

Spatial learning, structural reorganization, and the enhancement of synaptic communication in the hippocampal formation could all be considered endpoint measures of the influence of maternal care on hippocampal development and cognitive function, therefore, one must consider other factors that might drive the effects of maternal care. The hippocampus is one of the few regions of the brain that has regenerative capacity across the lifespan, thus, it is possible that maternal care during early life might affect proliferation and survival of neuronal progenitors in the proliferative zone of the hippocampus, and this effect might underlie most of the endpoint measures clearly elucidated by Liu et al (2000). The objective of these experiments was to examine the possibility that differences in neuronal proliferation and survival contribute to the effect of maternal care on cognitive function in adulthood. It was hypothesized that reduced maternal would lead to diminished neuronal proliferation and survival in the dentate gyrus of Low-LG offspring due to decreased levels of neurotrophic factor support in the hippocampus. (Portions of these data have been published in European Journal of Neuroscience, vol. 18(10), 2903-2909. With the exception of the nissl stain for pyknotic cell estimates, I performed all of the experiments presented in this chapter).

2.1. ABSTRACT- Maternal care during the first week of postnatal life influences hippocampal development and function (Liu et al., 2000). Offspring reared by mothers that exhibit increased levels of pup licking/grooming (LG) show increased hippocampal synaptic density and enhanced spatial learning and memory. Using 5-bromo-2'-deoxyuridine (BrdU), a thymidine analog incorporated into cells during

DNA synthesis, we examined the effects of early maternal care on hippocampal cell proliferation and neuronal survival in the rat. Twenty-four hours following injection on Day 7 of life there were no differences in BrdU labeling in the offspring of High- compared with Low-LG mothers, suggesting no maternal effect on the rate of proliferation at this age. However, 14 and 83 days following injection (postnatal day 21 and 90), the offspring of High-LG mothers had significantly more surviving BrdU labeled cells and BrdU/NeuN^{+} co-labeled neurons in the dentate gyrus subgranular zone and granule cell layer. At postnatal day 21, the offspring of High-LG mothers showed increased protein expression of bFGF and significantly decreased levels of pyknosis. These findings suggest an influence of maternal care on neuronal survival in the hippocampus. Conversely, at the same time point, there was a significantly higher level of hippocampal glial fibrillary acidic protein expression in the offspring of Low-LG mothers. These findings emphasize the importance of early maternal care for hippocampal development.

2.2. INTRODUCTION- Naturally occurring variations in maternal behavior, notably pup licking/grooming directly affect hippocampal development and cognition in the rat (Liu et al., 2000). The offspring of mothers that exhibit a higher frequency of licking/grooming (High-LG mothers) over the first week of postnatal life show increased hippocampal synaptic density and enhanced spatial learning and memory. The results of cross-fostering studies provide evidence for a direct relationship between maternal behavior and hippocampal development (Liu et al., 2000). These findings are consistent with recent reports showing that prolonged periods of mother-

infant separation in the rat during the first weeks of life are associated with evidence for increased apoptosis, decreased neurotrophic factor expression, and reduced mossy fiber density in adulthood (Lee et al. 2001; Roceri et al. 2002; Huot et al. 2002).

While separation/deprivation studies reflect the importance of mother-infant interactions for neuronal development, they do not directly address the issue of whether under normal conditions maternal care actively stimulates processes that foster survival and development within specific neuronal populations. The preliminary findings from a cDNA array study (Diorio et al. 2000) suggest that on postnatal day 8 of life there is increased expression of mRNAs for several neurotrophic factors, as well as mRNAs the protein products of which furnish metabolic support (i.e., glucose and fructose transporter, insulin and growth hormone receptors, etc.) in the offspring of High-LG mothers. Moreover, as adults, the offspring of High-LG mothers show decreased expression of the pro-apoptotic protein BAX and decreased levels of spontaneous apoptosis in the hippocampus (Weaver et al. 2002).

On the basis of these findings, we examined whether natural variation in maternal licking/grooming affects cell proliferation and neuronal survival in the hippocampus of the rat. We measured dentate gyrus cell proliferation and neuronal survival in the offspring of High- and Low-LG mothers using 5-bromo-2'-deoxyuridine (BrdU), a thymidine analog incorporated into cells during DNA synthesis. The phenotype of BrdU-labeled cells was defined using a neuronal nuclear marker (NeuN) for mature neurons, a neuronal marker (TUC-4) for immature, immediately post-mitotic neurons and glial fibrillary acidic protein (GFAP) as a

marker for glial cells. Basic fibroblast growth factor (bFGF) expression and the number of pyknotic cells in the hippocampus were also examined at postnatal day 21 to determine whether increased maternal licking/grooming early in life could provide an environment conducive for enhanced neuronal. The findings indicate a significant increase in bFGF expression and neuronal survival in the offspring of High-LG mothers, with no effects on cell proliferation, or the rate of neuronal maturation.

2.3. MATERIALS AND METHODS- *Animals.* The mothers were Long-Evans hooded rats born in our colony and derived from females obtained from Charles River Canada (St. Constant, Québec). Mothers and litters were housed in 46 cm x 18 cm x 30 cm 'Plexi-glass' cages with food and water provided *ad libidum.* The colony was maintained on a 12:12, light:dark schedule, with lights on at 0900. The animals underwent routine cage maintenance beginning on Day 12, but otherwise were not manipulated. All procedures were performed according to guidelines developed by the Canadian Council on Animal Care with protocols approved by the McGill Committee on Animal Care.

Maternal behaviour. The behaviour of each dam was observed for 5, 75-minute observation periods per day for the first 8 days postpartum using a procedure originally devised by Myers et al. 1989 (and see Liu et al., 1997; Francis et al., 1999a). Observers were trained using videotapes and still photography to a high level of inter-rater reliability (i.e., >.90). Observations were performed at 3 periods during the light phase 1000, 1400, 1600) and 2 periods during the dark phase of the Light:

Dark cycle (2000 and 0700). Within each observation period the behaviour of each mother was scored every 3 minutes (25 observations/period x 5 periods per day = 150 observations/mother/day) for the mother licking and grooming any pup (Myers et al., 1989; Liu et al., 1997).

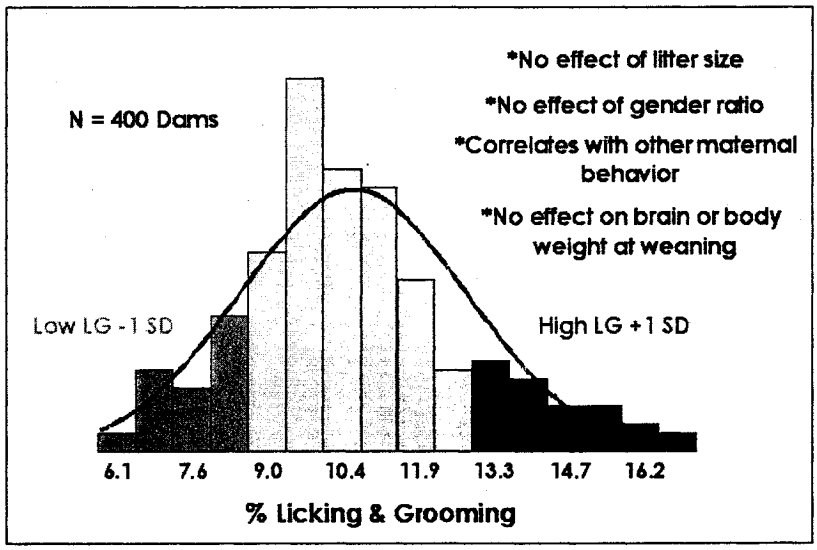


Figure 6. Representative histogram of the distribution of maternal LG (Champagne et al., 2003).

The frequency of maternal licking/grooming across a large number of mothers is normally and not bi-modally distributed (Champagne et al., 2003). Hence, the High- and Low-LG mothers represent two ends of a continuum, rather than distinct populations. In order to define these populations for the current study we observed the maternal behavior in a cohort of mothers, generally 30-40 dams with their pups, and devised the group mean and standard deviation for each behavior over the first 8 days of life as previously described (Francis et al. 1999; Liu et al. 2000). High-LG mothers were defined as females whose frequency scores for licking/grooming were greater

than 1 SD above the mean. Low-LG mothers were defined as females whose frequency scores for LG were greater than 1 SD below the mean (Figure 6).

BrdU labeling. On postnatal day 7, offspring of High- and Low-LG mothers from 3 litters were given a single injection of 5-bromo-2'-deoxyuridine (BrdU, 100mg/kg, i.p., Sigma, St. Louis, MO), dissolved in phosphate buffer (pH 8.5). To examine cell proliferation, 5 male rats from each group were sacrificed 24 h after BrdU injection. To examine cell differentiation and survival, 5 male rats from each group were sacrificed two weeks and three months after BrdU injection at P21 and P90.

Tissue preparation. For immunohistochemistry, male rats (n = 5/group) were deeply anaesthetized with Somnitol (50 mg/kg, i.p., Sigma, St. Louis, MO) and brains fixed by transcardial perfusion (50 ml NaCl, pH 7.4, followed by 2 x 50 ml 4% paraformaldehyde). Following a 24 h post fixation period in 4% paraformaldehyde and 30% sucrose, 50 μm coronal sections were cut in series on a vibratome and collected in a standard phosphate buffer (PBS 0.1 M, pH 7.4). The tissue was placed in cryoprotectant and stored at -20°C. For western blot, rats (n=5/group) were sacrificed by rapid decapitation, hippocampi dissected, and stored at -80°C until assay. At the time of sacrifice, brains were weighed and body weights were taken.

Immunohistochemistry. Free-floating sections were processed following a standard immunohistochemical procedure to determine BrdU labeling. Briefly, sections were treated with 60% formamide in SSC (30 min. at 50°C) followed by 2 N HCl (30 min.

at 37°C) and rinsed in a Borate buffer for 5 min (0.1M pH. 8.4). Sections were washed with PBS and preincubated for 45 min with PBS with 0.3% Triton X-100 and 3% horse normal serum (blocking solution), then incubated under agitation for 48 h at 4°C in primary rat monoclonal anti-BrdU antibody (1:1000 Accurate, Westbury, NY) in PBS containing 0.3% Triton X-100 and 3% horse normal solution. Serial sections from all animals were processed in parallel and visualized using donkey anti-rat CY3 fluorescent IgG antibody (2 h, 1:400, Jackson ImmunoResearch).

For double labeling, sections were prepared as above and incubated simultaneously for 48 h at 4°C in primary rat monoclonal anti-BrdU antibody (1:1000 Accurate, Westbury, NY) and mouse anti-GFAP antibody (2 h, 1:10000; Chemicon) or mouse anti-neuronal nuclei (NeuN) antibody (2 h, 1:1000; Chemicon) or rabbit anti- TUC-4 (Chemicon). Bound GFAP or NeuN antibodies were visualized with FITC anti-mouse antibody (2 h. 1:400; Chemicon), TUC-4 immunoreactivity was visualized using FITC anti-rabbit antibody (1:200; Chemicon). BrdU labeling was visualized using donkey anti-rat CY3 fluorescent IgG antibody (2 h, 1:400, Jackson ImmunoResearch).

Following the method of Lemaire et al. (1999), BrdU-labeled cells were counted in the subgranular zone (SGZ) and granule cell layer (GCL) of every sixth section (300 μm apart) throughout the dorsal hippocampus (6-7 sections per animal) under 40X objective magnification. The sections were counterstained and the surface area of the SGZ and GCL were calculated for each section by tracing each region using an MCID digital image analysis system. The numerical density of BrdU-labeled cells for each section was calculated by dividing the number of BrdU-labeled cells by

the SGZ and GCL sectional volume. Thus, the mean numerical density of BrdU-labeled cells was calculated for each animal. The total number of BrdU-labeled cells per dentate gyrus was calculated by multiplying the total numerical density of BrdU-labeled cells by the reference volume (using the Cavalieri estimation, $v_{ref} = a \times t \times s$; where a is the average area of the SGZ and GCL per dentate gyrus, t is the section thickness (assumed to be 50 μm), and s is the number of sections per animal; see Lemaire et al., 1999). Double-labeled cells were confirmed and photographed by confocal microscopy (Nikkon, PCM-2000). The number of co-labeled BrdU/NeuN$^+$ neurons was also calculated. A 2-way ANOVA (maternal care x age) was used to assess differences in the number of BrdU-labeled cells as well as co-labeled BrdU/NeuN$^+$ neurons.

Pyknotic cell estimates. Determination of pyknotic cell counts were made according to Frye and McCormick (2000). Briefly, whole brains were extracted (n = 8/group, as above), snap-frozen on dry ice, and stored at -80°C. Coronal sections (15 μm) corresponding to stereotaxic levels -2.30 – -3.80 mm from bregma (Paxinos and Watson, 1986) were collected onto gelatin-subbed slides and stored at -80 °C. Fresh frozen brain sections were processed for Nissl substance using cresyl violet. First, sections were briefly post-fixed in a solution of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer for 15 minutes, followed by 3 rinses with distilled water (2 minutes each). Next, sections were incubated in a 0.1% solution of cresyl violet for 2 minutes, rinsed twice in 95% ethanol, and twice in 100% ethanol (2 minutes per rinse). Sections were then cleared in xylene for 4 minutes before being coverslipped with

Permount. The absolute numbers of pyknotic cells were counted at the same sterotaxic level within a 100x visual field, over the subgranular zone and granule cell layer of the dentate gyrus. Cells that had a smaller volume had multiple, densely-stained clumps of chromatin, a condensed shriveled nucleus, and blebbing of the membrane were considered pyknotic. An observer blind to the experimental conditions performed the counts and statistical analysis was performed using Students t-test.

Gel Electrophoresis and Western Blot. Protein concentration of membrane preparations was determined according to the method of Bradford (Bradford, 1976). GFAP and bFGF protein expression was determined by electrophoresis and western blot analysis using mouse monoclonal anti-GFAP antibody (1:2000; Chemicon) and rabbit monoclonal anti-bFGF antibody (1:2000; Sigma). Individual samples, each constituting one male rat hippocampus (n = 5/group), were run on a single 15 well gel. Briefly, samples were prepared on ice (to a final volume of 15 µl), vortexed and denatured for 10 min. at 70^{o}C. 20% (15 well) gels (Novex) were run with 1X TBS-T and the proteins transferred onto PVDF membranes (Amersham). The membrane was blocked with 5 ml of 5% skim milk powder (Carnation) in TBS-T for 1 h at room temperature, washed with TBS-T for 5 min (3X) and incubated with 5 ml GFAP Ab (1:10000) in TBS-T in 0.5% skim milk powder for 24 h at 4^{o}C. The membrane was washed with TBS-T (3X), incubated for 1 h with anti-mouse horseradish peroxidase linked secondary Ab in TBS-T (1:2500) and washed in TBS-T for 10 min (5X) and 20 min (1X). Signal detection was performed using an enzyme chemiluminescence

kit (ECL, Amersham). Optical density readings of the film were taken using the MCID (St Catherines, Ontario) image analysis system. The membrane was then stripped and probed with anti-tubulin (Sigma, St. Louis, 1:2000). bFGF expression was analysed by 2-way (maternal care x isoform) ANOVA and GFAP protein expression was analysed by students T-test. For analysis, we used the ratio of the relative optical density for bFGF or GFAP over the relative optical density for tubulin, thus providing an internal control for each individual sample.

2.4. RESULTS- Male pups were selected based on observations of maternal behavior of cohort of lactating mothers as described above. The mean ± SD for the High- (14.0 ± 1.3) and Low- LG (6.7 ± 0.7) mothers for the present cohort (overall mean ± SD = 10.5 ± 2.3) lies well within the range of our previous studies (see Champagne et al. 2003). Across the population of lactating mothers observed within our lab from 1998-2003 the mean ± SD for High-LG mothers was 14.7 ± 1.3 and for Low-LG dams it was 7.0 ± 1.1). There was no significant effect of maternal care on BrdU-labeling on postnatal day 8 suggesting that maternal care did not alter neuronal proliferation at this age (data not presented). At both postnatal days 21 and 90, there was a significant main effect of maternal care ($F_{1,16} = 64.94$, $p < .0001$) on the number of surviving BrdU-labeled cells in the dentate gyrus, with a significantly greater number of BrdU-labeled cells in the offspring of High- compared to Low-LG mothers (Figure 7 and 9). There was a significant main effect of age ($F_{1,16} = 16.26$, $p < .001$) on surviving BrdU-labeled cells in the dentate gyrus; the number of surviving BrdU-labeled cells decreased in both groups from P21 to P90.

Figure 1a)

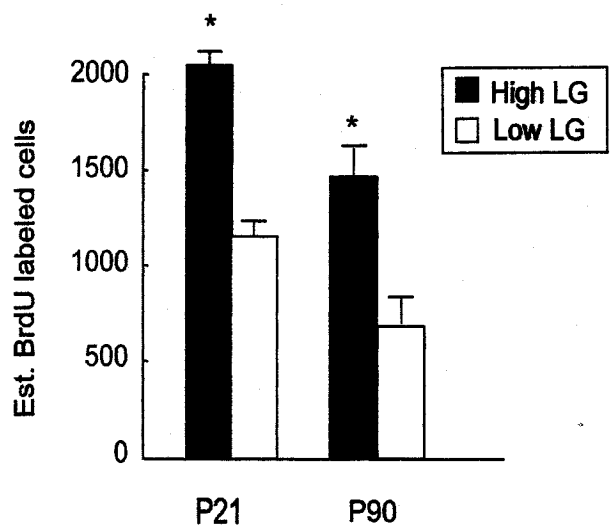


Figure 1b)

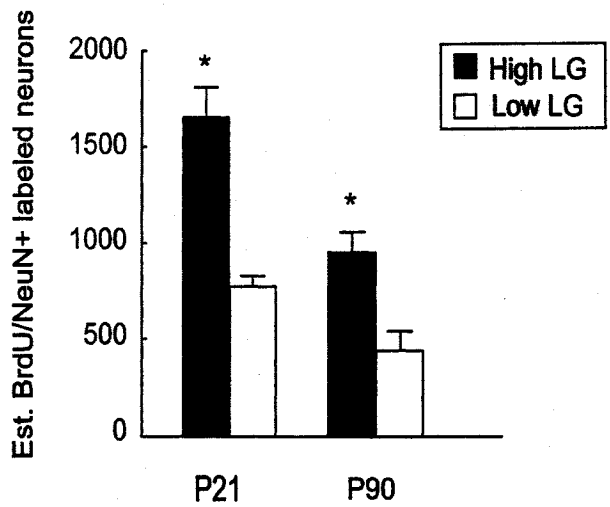
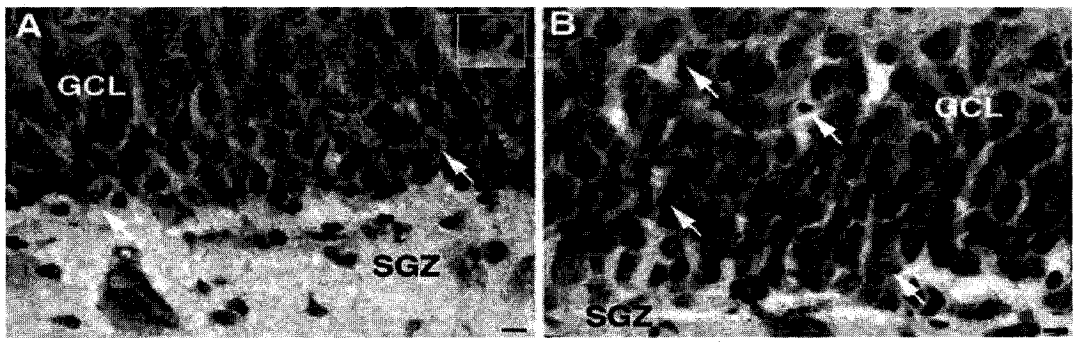


Figure. 7. (a) Mean ± SEM estimated number of BrdU-labeled cells in the dentate gyrus of day 21 and 90 offspring of High- and Low-LG mothers (* $p < .0001$). (b) Mean ± SEM estimated number of co-labeled BrdU/NeuN$^+$ neurons in the dentate gyrus of day 21 and 90 offspring of High- and Low-LG mothers (* $p < .0001$) (Bredy et al., 2003).



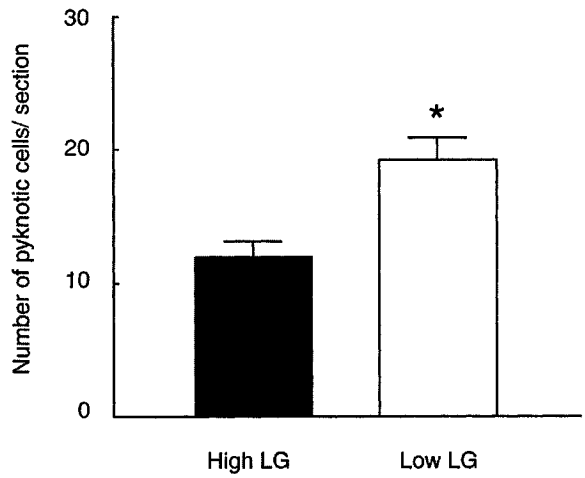


Figure 8. Mean ± SEM estimated number of pyknotic cells in the dentate gyrus of day 21 offspring of High- and Low-LG mothers (* $p < .01$). Panels A and B. Light microscopic image (1000x) of cresyl violet stained pyknotic cells in the P21 offspring of (a) High- and (b) Low-LG mothers (scale: 20 μm). Inset in panel (a) is an enhanced image of what would typically be considered a pyknotic cell (Bredy et al., 2003).

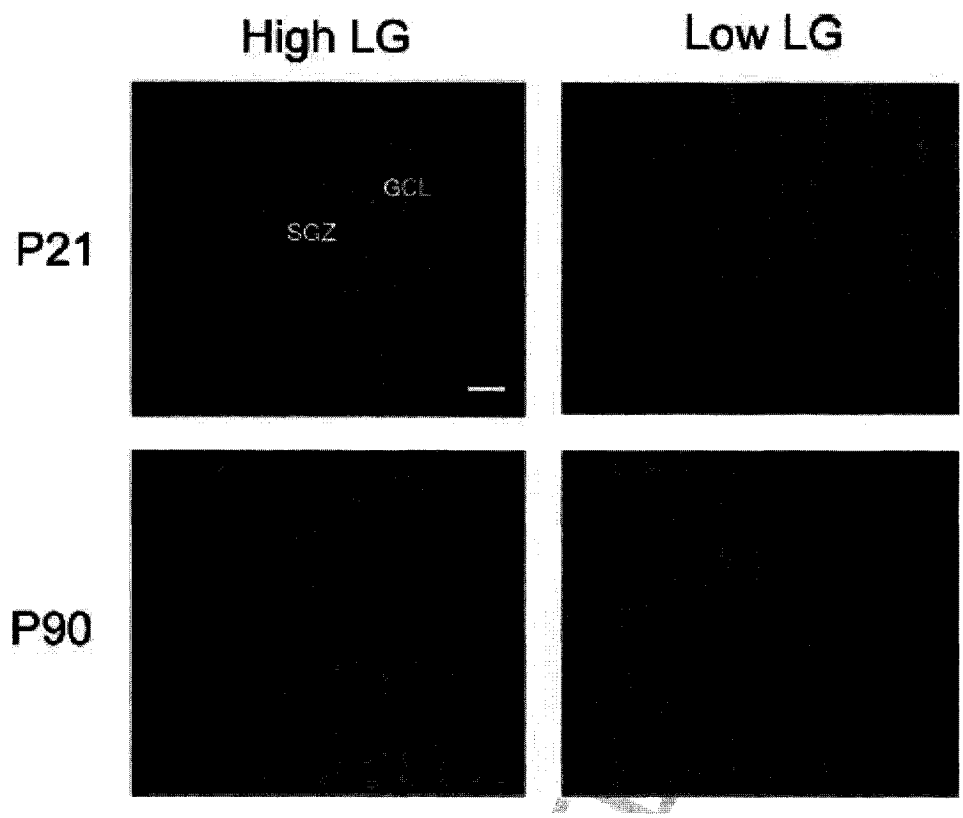


Figure 9. Confocal scanning laser micrograph of BrdU labeled cells in P21 and P90 offspring of High- and Low-LG mothers (scale: 20 μm) (Bredy et al., 2003).

Double-labeling with antibodies for BrdU and NeuN revealed a significant main effect of maternal care ($F_{1,16} = 60.81$, $p < .0001$) on the number of co-labeled BrdU/NeuN$^+$ neurons in the dentate gyrus of the offspring of High- compared to Low-LG mothers (Figures 7 and 10a). There was also a significant main effect of age ($F_{1,16} = 60.81$, $p < .0001$) as the number of co-localized BrdU/NeuN$^+$ labeled neurons in the dentate gyrus decreased in both groups (Figure 10b). At P21, there was a highly significant effect ($t_{14} = 3.60$; $p < .01$) of maternal care on the number of pyknotic cells (Figures 7 and 8), as the offspring of High-LG mothers had a reduced level of pyknosis in the dentate gyrus. There was no effect of maternal care on TUC-4 expression (>5% colocalization (Figure 10c).

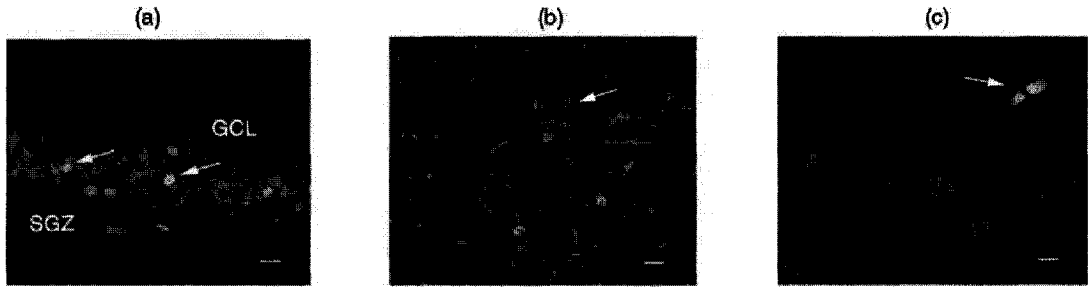


Figure 10. (a) Confocal scanning laser micrograph where approximately 60% of BrdU labeled cells were confirmed to be co-labeled BrdU/NeuN$^+$ neurons in P21 offspring of High- and Low-LG mothers (scale: 20 μm), (b) BrdU labeled cells co-labeled for BrdU/GFAP$^+$ astrocytes (> 10%) in P21 offspring of High- and Low-LG mothers (scale: 20 μm), (c) Very few BrdU labeled cells (>5%) co-labeled with Tuc-4 in P21 offspring of High- and Low-LG mothers (scale: 10 μm) (Bredy et al., 2003).

At postnatal day 21, there was a significant main effect of maternal care ($F_{1, 23}$ = 26.14, $p < .001$) on bFGF protein expression in the offspring of High- compared to Low-LG mothers (Figure 11). Levels of bFGF-IR were significantly higher in the offspring of High- compared with Low-LG mothers for all three neuronal isoforms. There was also a significant main effect of isoform ($F_{2,23}$ = 15.31, $p < .0001$) as the 18 and 20 kDa isoforms of bFGF protein were increased in both groups compared to the 22 kDa isoform.

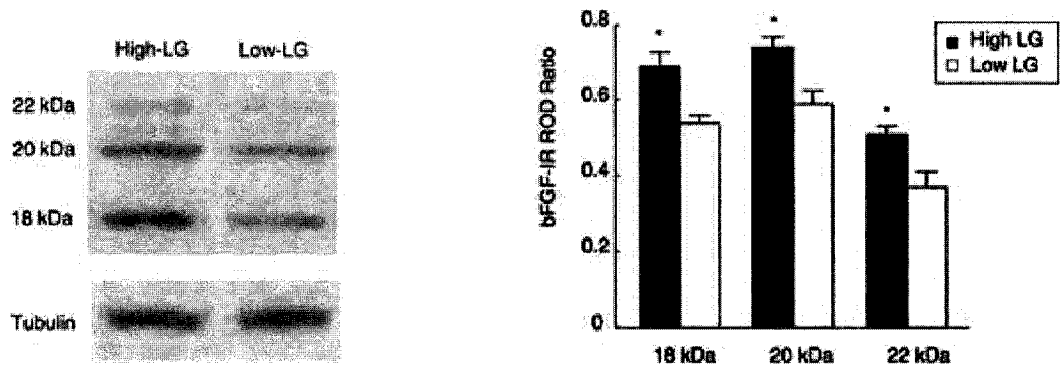


Figure 11. Mean ± SEM whole hippocampal membrane homogenate levels of the 18, 20 and 22 kDa isoforms of bFGF in the day 21 offspring of High- and Low-LG mothers, expressed as ratio of relative optical density (ROD) units. Left panel shows a representative result from a Western blot with α-tubulin used to control for loading errors (* $p < .001$) (Bredy et al., 2003).

There was little co-localization (>10%) of BrdU with GFAP (see Figure 9b) in either group. However, there was an unexpected increase in GFAP-IR in the day 21 offspring of Low-LG mothers (Figure 12a). The increase in GFAP expression was confirmed by Western blot ($T_8 = 2.27$, $p < .05$; Figure 12b). Brain and body weights did not differ between groups at P21 or P90 (data not presented).

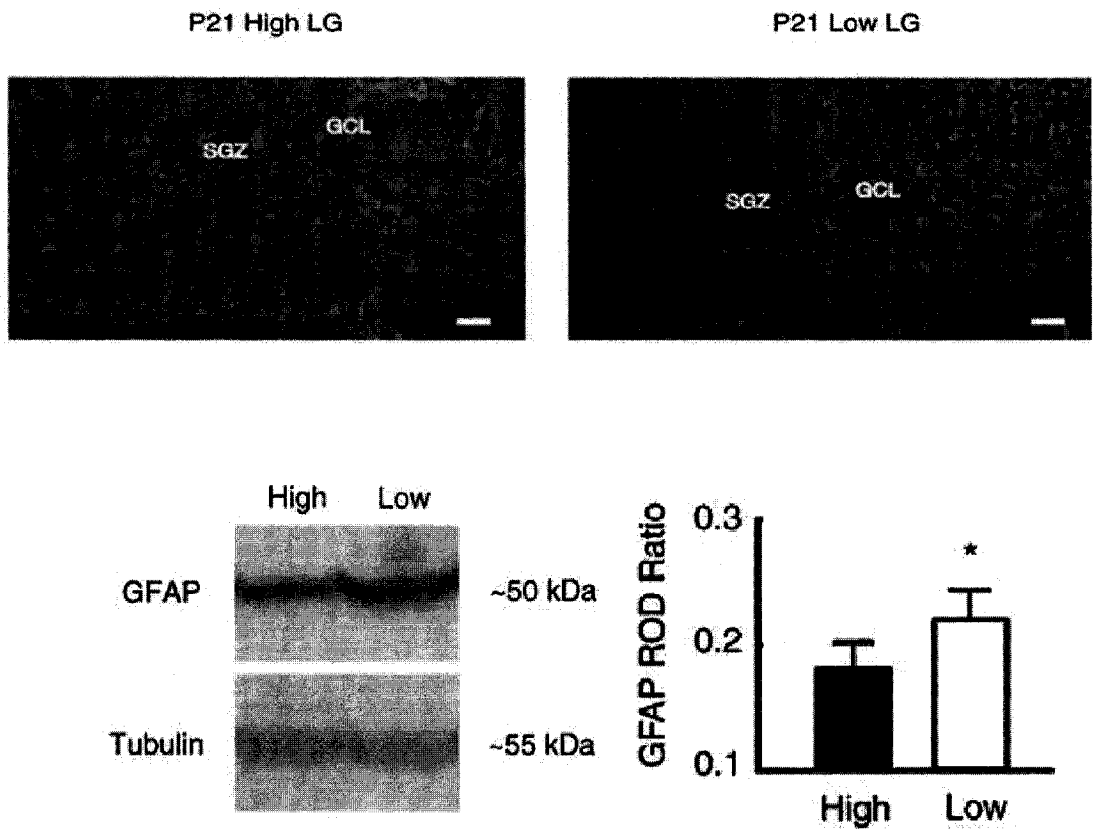


Figure 12. (a) Confocal laser scanning microscopic image of GFAP-labeled cells (FITC, 1:400) in the dentate gyrus (scale bar: 20 μm). (b-c) Hippocampal membrane homogenates processed for Western blot analysis confirmed increased GFAP-like immunoreactivity in the day 21 Low-LG offspring (expressed as a ratio of ROD with α-tubulin to control for loading errors (* $p < .05$) (Bredy et al., 2003).

2.5. DISCUSSION- Maternal care appears to directly influence hippocampal development and thus contributes to individual differences in spatial learning and memory (Liu et al. 2000). In the present study, variations in maternal care did not affect cell proliferation or the rate of neuronal maturation, but were associated with differences in hippocampal neuronal survival that appeared to persist into adulthood. Finally, we observed an unexpected increase in GFAP immunoreactivity in the dentate gyrus of Low-LG mothers.

Hippocampal bFGF protein expression was increased in the offspring of High- compared with Low-LG mothers at postnatal day 21 (Figure 11). This time point corresponds to the end of weaning and a period of rapid hippocampal development (Altman and Bayer, 1990). The neuronal isoforms of bFGF have both autocrine and paracrine effects that promote neuronal proliferation, differentiation and survival (Ray et al., 1993; Wagner et al., 1999; Williams et al., 1994; Aoyagi et al., 1994; Walicke, 1988; Delrieu, 2000). Furthermore, an injection of bFGF at the time of birth is associated with increased neuronal survival at the time of weaning, and under in vitro conditions bFGF increases synaptogenesis in hippocampal neurons (Cheng et al., 2002; Li et al., 2002). We have previously shown that, as adults, the offspring of High-LG mothers show increased hippocampal synaptogenesis (as measured by synaptophysin protein expression) and reduced hippocampal apoptosis compared with the offspring of Low-LG mothers (Liu et al., 2000; Weaver et al., 2002).

Taken together with the 2-fold increase in the level of hippocampal pyknosis apparent in the offspring of Low-LG mothers (Figures 8), these findings suggest that maternal care provides an environment conducive for neuronal survival early in life.

Further evidence for this idea emerges from the effect of maternal care on the number of neonatally BrdU-labeled cells in the P21 and P90 offspring (see Figures 7 and 9). We propose an influence of maternal care on neuronal survival and, subsequently, on the development of functional circuitry within the hippocampus, which might be related to previously observed effects of maternal care on hippocampal-dependent learning in adulthood (Liu et al. 2000).

Brain-derived neurotrophic factor (BDNF) expression in the hippocampus is also increased in the neonatal offspring of High LG mothers (Liu et al., 2000). cDNA array studies (Diorio et al., 2000) revealed an increase in the expression of genes encoding for BDNF and bFGF. Maternal care also influences the expression of the NR2A and NR2B subunits of the NMDA receptor as well as NMDA receptor binding capacity (Liu et al., 2000; Bredy et al., 2003). In agreement with the results of studies on the effect of maternal separation/deprivation (Lee et al. 2001; Huot et al. 2002; Roceri et al., 2002), these findings support the idea that maternal behavior actively stimulates hippocampal development in the offspring through systems known to mediate experience-dependent neural plasticity (Meaney et al., 2001). The results of the present study, together with that of Liu et al. (2000), suggest that more subtle variations in early experience, involving differences in mother-infant interactions, produce substantial effects on hippocampal development even in the absence of any obvious source of adversity.

A surprising finding was that of the significant increase in GFAP expression in the day 21 offspring of Low-LG mothers (Figure 12). The location and nature of the staining is consistent with an enhanced number of mature astrocytes. Recent

reports suggest that GFAP expressing astrocytes in the SGZ may actually serve as neuronal precursors and actively promote synaptic maintenance and development (Seri et al., 2001; Ullian et al. 2001). Thus, an increased population of astrocytes in the offspring of Low-LG mothers at the time of weaning may have long lasting effects on hippocampal plasticity that could influence cognitive function across the lifespan.

A crucial question that comes to mind when interpreting these data is whether GFAP-labeled astrocytes are in fact stem cell progenitors, or simply reactive glia. Alvarez-Buylla and Lim (2004) describe several proteins that uniquely interact with a subpopulation of astrocytes within the SGZ. For example, LeX, a carbohydrate moiety, is expressed in blood vessels of the SGZ where it is associated with a specific population of astrocytes thought to have progenitor-like characteristics (Capela and Temple, 2002). Eph/ephrin signaling molecules are also associated with astrocytes in the SGZ, and icv infusion of EphB2.3 causes these astrocytes to proliferate (Conover et al., 2000). Currently, there are no clear markers for defining which GFAP expressing astrocytes will undergo asymmetric division to generate new neurons. Seri et al (2000) describe these neurogenic intermediates as B cells within the radial glia-astrocyte-neuron lineage, and identify them based on their morphological characteristics. There is one study that has suggested that some GFAP-labeled astrocytes may also express nestin, a marker for stem cells within the CNS (Lendahl et al., 1990). In order to clarify whether the differences we observe in neuronal survival between the High-LG and Low-LG offspring are the result of an increased population of neurogenic astrocytes, we could perform triple labeling studies for

BrdU, GFAP and nestin, in combination with a thorough morphological characterization of every newborn neuron. Further, we could then design a study that demonstrates an interaction between these cells and LeX or EphB within the SGZ.

2.6. CONCLUDING REMARKS- In summary, maternal care increased hippocampal neuronal survival in the offspring of High- compared with Low-LG mothers with no differences in cell proliferation or rate of neuronal maturation in the neonate. Such effects may contribute, in part, to the influence of early maternal care on the development of hippocampal synaptic density, and markers of synaptic communication previously demonstrated by Liu et al (2000). The findings emphasize the importance of environmental stimulation early in life for cognitive function in adulthood. The critical question and the focus for the remainder of this thesis is to determine if the effect of reduced maternal care on hippocampal development can be reversed through exposure to environmental enrichment during adolescence, and if this is the case, to determine the neural mechanisms that mediate the effect.

CHAPTER 3- PARTIAL REVERSAL OF THE EFFECT OF MATERNAL CARE ON COGNTIVE DEVELOPMENT IN THE RAT

3. RATIONALE- Both maternal care and postweaning environmental enrichment affect spatial learning, hippocampal synaptic density and neuronal survival in the dentate gyrus (Saito et al., 1994; Nilsson et al., 1997; Liu et al., 2000; Bredy et al., 2003). Thus, the main objective of the following experiments was to determine whether the effect of reduced maternal care on cognitive function could be reversed by exposure to environmental enrichment during adolescence. We attempted to elucidate some of the underlying neural mechanisms mediating the effects by examining several putative targets known to be involved in hippocampal-dependent learning such as glutamate receptor binding and functional synaptic plasticity in the dentate gyrus. We hypothesized that environmental enrichment would reverse the effect of reduced maternal care on hippocampal-dependent learning and that these effects would be accompanied by changes in NMDA and AMPA receptor binding and long-term potentiation in the dentate gyrus. (Portions of these data have been published in Neuroscience, vol. 118, pp. 571-576. With the exception of running Morris water maze, I performed all of the experiments for the data presented in this chapter).

3.1. ABSTRACT- Maternal care influences hippocampal development in the rat. The offspring of mothers that exhibit increased levels of pup licking/grooming (High-LG mothers) show increased hippocampal NMDA receptor binding and enhanced hippocampal-dependent spatial learning. In these studies we examined whether

environmental enrichment from Days 22 to 70 of life might reverse the effects of low maternal care. Environmental enrichment eliminated the differences between the offspring of High- and Low-LG mothers in both Morris water maze learning and object recognition. However, enrichment did not reverse the effect of maternal care on long-term potentiation in the dentate gyrus nor on hippocampal NMDA receptor binding. In contrast, peripubertal enrichment did reverse the effects of maternal care on hippocampal AMPA receptor binding. These findings provide evidence for a substantial capacity for the reversal of the effects of low maternal care at the level of function that is due, in part at least, to compensatory effects associated with peripubertal enrichment.

3.2. INTRODUCTION- Parental care influences cognitive development in humans, primates and rodents (Tamaroff et al., 1986; Kraemer, 1997; Liu et al., 2000). For the rat pup, the dam is the most pervasive and dynamic source of sensory stimulation in the postpartum environment and mother-pup interactions influence hippocampal synaptic development and function (Liu et al., 1997; Liu et al., 2000). The tactile stimulation derived from maternal licking/grooming and arched-back nursing appears to be critical for neural development. The offspring of mothers that show increased licking/grooming and arched-back nursing (i.e., High-LG mothers) exhibit increased NMDA receptor and neurotrophic factor expression as well as enhanced spatial learning and memory (Liu et al., 2000). These effects of maternal care are reversed with cross-fostering, such that, as adults, the biological offspring of Low-LG mothers reared by High-LG dams are indistinguishable from the normal offspring of High-LG mothers on measures of hippocampal development or spatial learning and memory (Liu et al. 2000).

Environmental influences on hippocampal development are not limited to the mother-infant interaction during the postnatal period. Hebb (1949) described how environmental enrichment throughout the peripubertal period enhanced maze learning in the rat. Rats exposed to environmental enrichment exhibit increased hippocampal nerve growth factor (NGF) and NGFI-A (an activity-dependent transcription factor) mRNA expression as well as improved performance in the Morris water maze (Pham et al., 1999; Olsson et al., 1994). Environmental enrichment also enhances neuron proliferation in the dentate gyrus (DG) of the hippocampus in rats and mice (Nilsson et al., 1999; Kempermann et al., 1997).

Considering the evidence for the positive effects of environmental enrichment, we hypothesized that the effect of reduced maternal stimulation on hippocampal development and learning and memory could be reversed by environmental enrichment and whether such effects might reflect reversal at the level of putative underlying mechanisms, including glutamate receptor expression. The Day 22 offspring of High- or Low-LG mothers were reared from weaning until Day 70 under conditions of environmental enrichment or standard housing and tested between Day 120 and Day 140 of life.

3.3. METHODS AND MATERIALS- *Animals.* The mothers were Long-Evans hooded rats born in our colony and derived from females obtained from Charles River Canada (St. Constant, Quebec). Mothers and litters were housed in 46 cm x 18 cm x 30 cm "Plexiglass" cages with food and water provided ad libidum. The colony was maintained on a 12:12 light:dark schedule with lights on at 0800. The animals underwent routine cage

maintenance beginning on Day 12, but were otherwise not manipulated. All procedures were performed according to guidelines developed by the Canadian Council on Animal Care with protocols approved by the McGill Committee on Animal Care.

Maternal behavior. Please refer to that described in Chapter 2.

Postweaning housing conditions. On Day 22 of life, the male offspring from five litters each of High and Low mothers were randomly assigned to either environmental enrichment or standard housing conditions. Animals housed under conditions of environmental enrichment were placed into groups of 8 animals living within a series of large 60 x 30 x 60 cm cages interconnected with a burrow system and filled with toys that were replaced regularly. Standard lab conditions were defined as 2 animals housed in a 20 x 40 30 cm clear plastic cage. Animals were removed from these conditions on Day 70 and housed two-per cage until testing began on approximately Day 120. During behavioural testing, all subjects were changed to single housing and remained this way until the end of the experiment.

Morris Water Maze. Rats were required to locate a submerged platform (15 x 15 cm) that was 1 cm below the water line in a 1.5 m-diameter pool using only ambient spatial cues available within the testing room (Morris, 1982). The water was made opaque by a layer of white polypropylene pellets that floated on the surface. Between 1000 and 1600, the rats were given 20 trials over 2 consecutive days with reversal of platform location on day 2 (10 trials per day, minimum 20 minutes inter-trial interval) with the platform submerged. At the end of each testing day, subjects were given a

probe trial (60 s) in which the platform was removed to determine spatial bias for platform location. For all tests, search time (s), thigmotaxis (time spent in the pool periphery within less than 20 cm of the pool wall; Hoh et al., 1999) and time spent in quadrant (s) were recorded using the Poly-Track video tracking system (San Diego Instruments). Latency and thigmotaxis, were analysed by between within ANOVA (group x housing x time)

Object Recognition Test. The object recognition test was performed according to Ennaceur and Delacour (1988), with modification. The testing apparatus was a standard open field box ($50cm^3$), painted black with bedding material covering the floor. The rats were habituated to the testing apparatus with three, daily 5 min. sessions. The memory test consisted of a sample phase followed by a choice phase with a 15 min. inter-trial interval. During the sample phase, the rat was allowed to explore two identical objects for 5 min. and then returned to its home cage. Both objects were removed during the 15 min. interval and replaced with one identical and one novel object. The rat was then placed back in the box for a 5 min. choice phase. Both objects and object location were counterbalanced in order to remove object and location preference effects. The experimenter was blind to the identity of the subjects during the experiment and each trial was videotaped for offline analysis. Exploration of an object was defined as directing the nose towards the object at a distance of 1 cm and/or touching the object with nose and paws. In order to obtain a measure of object discrimination, four measures were recorded; a) time spent exploring each object during the sample phase (T1a, T1b) and b) time spent exploring each object during

the choice phase (T2a, T2n). For the result, discrimination was determined using the following formula; (T2n-T2a)/ T2n+T2a) and analysed by 2-way ANOVA (group x housing).

Tissue Preparation. Brains were obtained from adult (approximately 100 days of age) offspring of High- and Low-LG mothers by rapid decapitation, snap frozen in -70°C isopentane and stored at -80°C. Frozen coronal brain sections (16μm) were cut on a cryostat, thaw-mounted on polylysine-coated slides and stored at -80°C until assay.

Electrophysiology. Rats were anaesthetised (Somnotol, 65mg/kg, i.p.) and implanted with chronic stimulating and recording electrodes using stereotaxic techniques under aseptic conditions. Briefly, stimulating electrodes were implanted unilaterally in the medial perforant path with recording electrodes placed in the ipsilateral dentate hilus. Ground screws were placed in the skull along with two anchoring screws. Final positioning of the electrodes was determined during surgery by evoking field potentials using single test pulses. Rats were given a minimum of one week to recover.

Field potentials (EP) were evoked in unanaesthetised, freely moving rats (in a quiet state of immobility) with single diphasic (0.1msec/phase) test pulses delivered a minimum of 15 s apart (Hoh et al., 1999). Input-output (IO curve) determination (five to seven test pulse intensities) was followed by three days of stable baseline recording of EPs induced by two test pulses of varying intensity (minimum and 80% of maximum). LTP was induced via five high-frequency trains, approximately 20 s

apart (each train was 50 diphasic pulses, 0.1 ms/phase, 400Hz, near maximal input-out response) and applied to the perforant path (Cain et al., 1997). Averaged responses to the same test pulse (10 sweeps) were obtained immediately, 1 hr, and 24 hr post LTP induction. Electrographic activity at the recording site was continuously monitored to detect any after discharge (AD) that may have compromised the recording. Between within ANOVA was used to analyse the rising phase of the averaged field EPSP (slope) as well as the population spike amplitude (PSA). Upon completion of the experiment, rats were anaesthetised (somnotol, 65mg/kg, i.p.) and transcardially perfused with 10% formalin. The brains were cut in 40 μm sections, counterstained with cresyl violet and electrode placement was confirmed.

Glutamate Receptor Autoradiography. NMDA receptor binding sites were labeled with ^{3}H-MK-801 (21.7 Ci/mmol, RBI) according to Glazewski et al. (1993). Slide-mounted sections were thawed for 30 min at 4°C and then pre-incubated in 5mM Tris-HCl, pH 7.4, buffer for 10 min at 5°C. Incubation was performed for 1 h at 20°C in the same buffer containing 5μM spermidine, 5μM glycine, 5μM glutamate and 3nM ^{3}H-MK-801. Non-specific binding was assessed on adjacent sections with incubation buffer containing 100 μM cold MK-801. After incubation, slides were rinsed in 3 x ice-cold buffer (30 s), dH_20 and 2.5% glutaraldehyde in acetone and then dried overnight. The incubated slides along with [^{3}H] standards (Amersham) were exposed to tritium sensitive film (Hyperfilm, Amersham, Toronto, Ontario) for 3-4 weeks at 5°C. Regional NMDA receptor binding was analysed using an MCID

Image Analysis system (MCID, St. Catherines, Ontario) and statistical analysis performed using a 3-way ANOVA (maternal care by housing by region).

AMPA receptor binding sites were labeled with ^{3}H-AMPA (42.2 Ci/mmol, RBI) according to Le Jeune et al. (1996). Slide-mounted sections were thawed for 30 min at 4°C, and then pre-incubated in 50 mM Tris-acetate, pH 7.2, buffer for 1 h at 4°C. Incubation was performed for 30 min at 4°C in the same buffer containing 100 μM potassium thiocyanate and 50 nM ^{3}H-AMPA. Non-specific binding was assessed on adjacent sections with incubation buffer containing 1 mM cold L-glutamic acid. After incubation, slides were rinsed in ice-cold buffer and dH_20 and dried overnight. The incubated slides along [3H] standards (Amersham) were exposed to tritium-sensitive film (Hyperfilm, Amersham, Toronto, Ontario) for 6 weeks at 4oC. Regional AMPA receptor binding was analysed using an MCID Image Analysis system (MCID, St. Catherines, Ontario) and statistical analysis performed using a 3-way ANOVA (maternal care by housing by region).

3.4. RESULTS- *Morris Water Maze*. Overall, there was a significant main effect of maternal care ($F_{1,64} = 4.57$, $p < .05$), housing ($F_{1,64} = 34.07$, $p < .0001$) and day ($F_{1,64} = 54.59$, $p < .0001$) on latency to find the platform. Enriched rats showed significantly shorter latencies than standard-housed rats and the latency to find the platform decreased for all groups across days. Post-hoc analysis (Tukey/Kraemer test) showed that enriched rats had a shorter latency to find the platform on trial blocks 2-5 (all p's < .01). There was a significant maternal care by housing interaction ($F_{1,64}$= 3.92, $p < .05$). The standard housed offspring of High-LG mothers showing a

significantly shorter search time than the offspring of Low-LG mothers. Among animals reared under conditions of enrichment this difference was eliminated (Figure 13). Simple effects analysis revealed that in the standard housed group, the offspring of High-LG mothers had shorter latency to find the platform on trial blocks 2 and 4 (all p's < .05). There were no differences in quadrant dwell time during the probe trial at the end of day 1, indicating that all subjects learned to find the escape platform (data not shown).

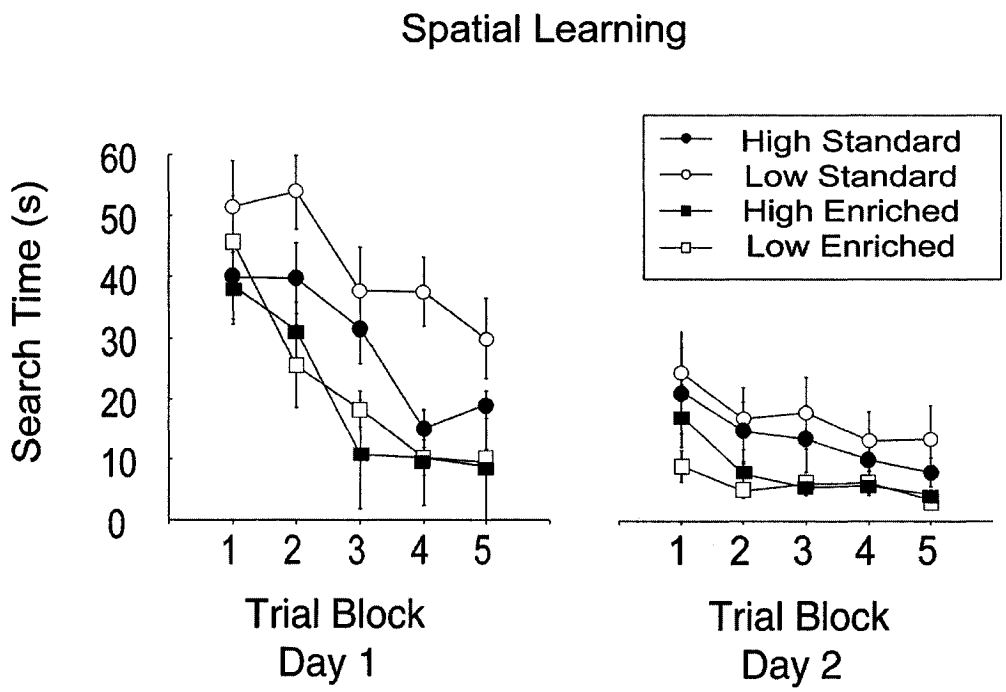


Figure 13. Mean ± SEM latency to find platform (s) (n = 8-10/group) on days 1 and 2 of Morris water maze training for adult offspring of High- and Low-LG mothers reared under standard or enriched housing conditions (Bredy et al., 2003).

Object Recognition Test. There was a significant main effect of maternal care ($F_{1,34} = 7.93$, $p < .01$) and housing ($F_{1,34} = 11.04$, $p < .01$) on performance in the object discrimination test. Overall, the offspring of High-LG mothers spent more time exploring the novel object during the choice phase of the object recognition test than did the offspring of Low-LG mothers. Most importantly, there was a significant maternal care by housing interaction ($F_{1,34} = 4.94$, $p < .05$) with the standard housed offspring of High-LG mothers performing significantly better than the standard housed offspring of Low-LG mothers. This difference was eliminated under conditions of enrichment with a reversal in the performance of the offspring of Low-LG mothers such that the offspring of Low-LG mothers reared under conditions of environmental enrichment did not differ from either group of High-LG offspring (Figure 14).

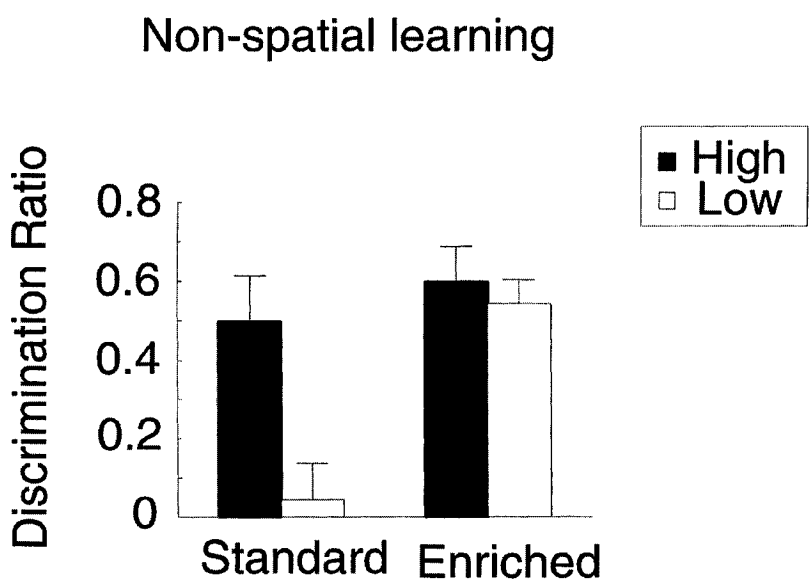


Figure 14. Mean ± SEM discrimination ratio (n = 8-10/group) between novel and similar objects during the choice phase of the object recognition test (Bredy et al., 2003).

Electrophysiology. There were no effects of maternal care or housing on the averaged field EPSP (data not shown). The offspring of High-LG mothers showed a trend towards greater population spike amplitude (PSA) at 0, 1 and 24 h post LTP ($F_{1,15} = 3.35$, $p < .10$). Collapsing the data revealed a significant main effect of maternal care ($F_{1,15} = 5.21$, $p < .05$) on average PSA. The offspring of High-LG mothers had a greater average PSA than the offspring of Low-LG mothers, regardless of housing condition (Figure 15).

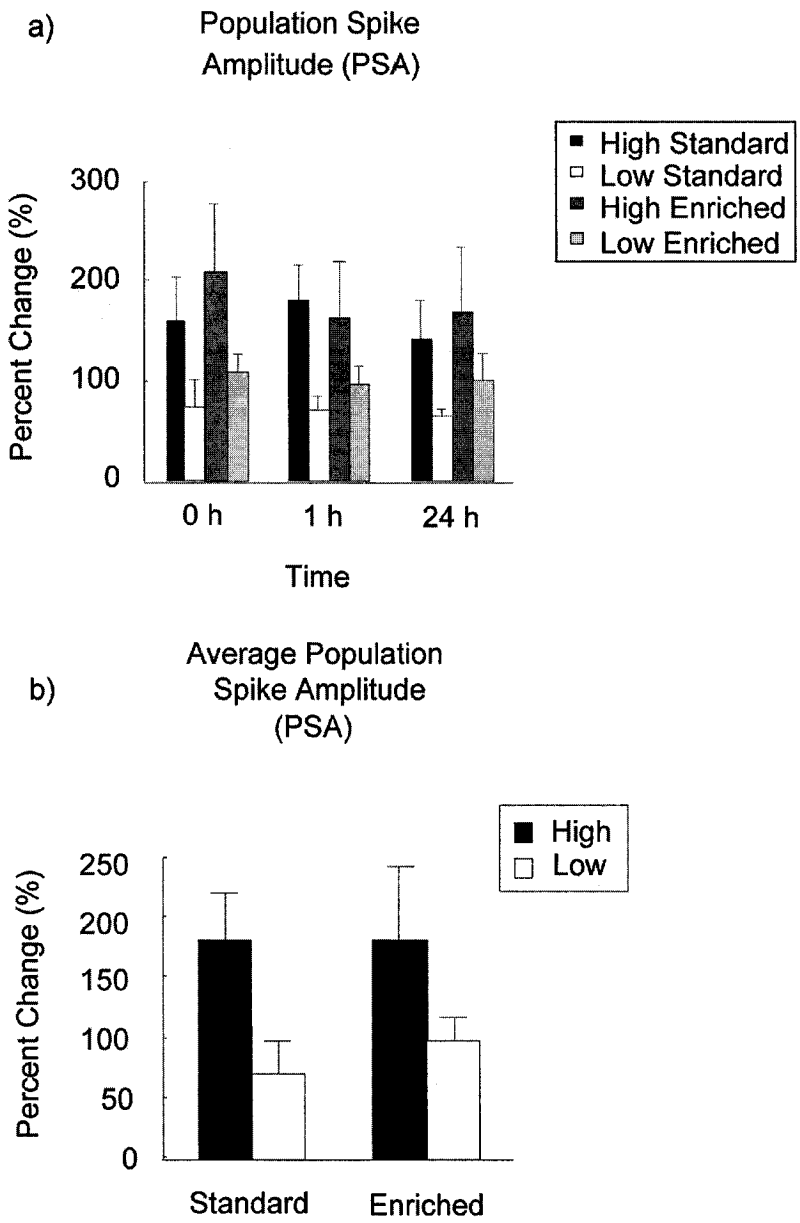


Figure 15. Mean ± SEM population spike amplitude (n = 4-5/group) for adult offspring of High- and Low-LG mothers reared under standard or enriched housing conditions (Bredy et al., 2003).

Glutamate Receptor Autoradiography.

^{3}H-MK-801 Binding. There was a significant main effect of maternal care ($F_{1,90}$ = 79.52, $p < .0001$) and region ($F_{1,90}$= 40.70, $p < .0001$) on ^{3}H-MK-801 binding. The offspring of High-LG mothers had higher ^{3}H-MK801 binding in all hippocampal regions, regardless of housing condition and ^{3}H-MK801 binding was highest in the CA1 and lowest in the DG (Figure 16). Environmental enrichment had no effect on ^{3}H-MK801 binding in either group.

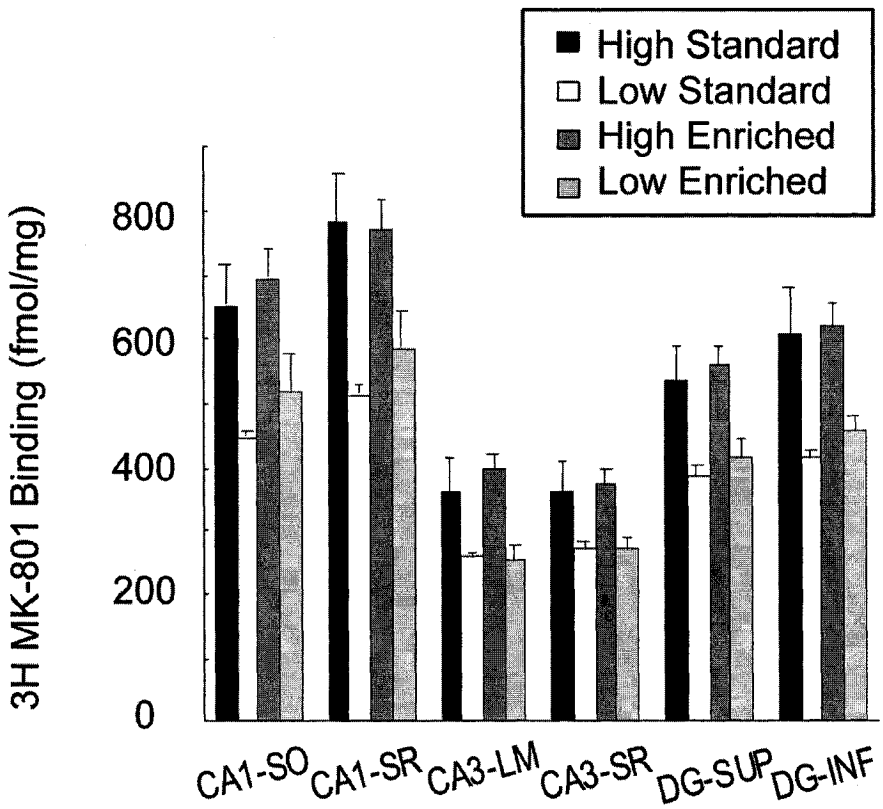


Figure 16. Mean ± SEM hippocampal specific [^{3}H] MK-801 binding (n = 4-5/group) for adult offspring of High- and Low-LG mothers reared under standard or enriched housing conditions. (SO- stratum oriens, SR- stratum radiatum, INF- inferior blade, SUP- superior blade) (Bredy et al., 2003).

^{3}H-AMPA Binding. There was a significant main effect of maternal care (F1,78 = 70.12, p < .0001), housing (F1,78 = 25.50, p < .0001), and region (F1,78 = 2.53, p < .05) on ^{3}H-AMPA binding. ^{3}H-AMPA binding was highest in the CA1 and lowest in the CA3. The offspring of Low-LG mothers had higher ^{3}H-AMPA binding in all regions, regardless of housing condition and environmental enrichment decreased ^{3}H-AMPA binding in all regions in both groups. Simple effects analysis of maternal care at housing/region revealed that under conditions of enrichment, ^{3}H-AMPA binding did not differ between the offspring of High- and Low-LG mothers in the CA1 stratum radiatum or inferior blade of the dentate gyrus (F1,78 = 2.50, p >.05) (Figure 17).

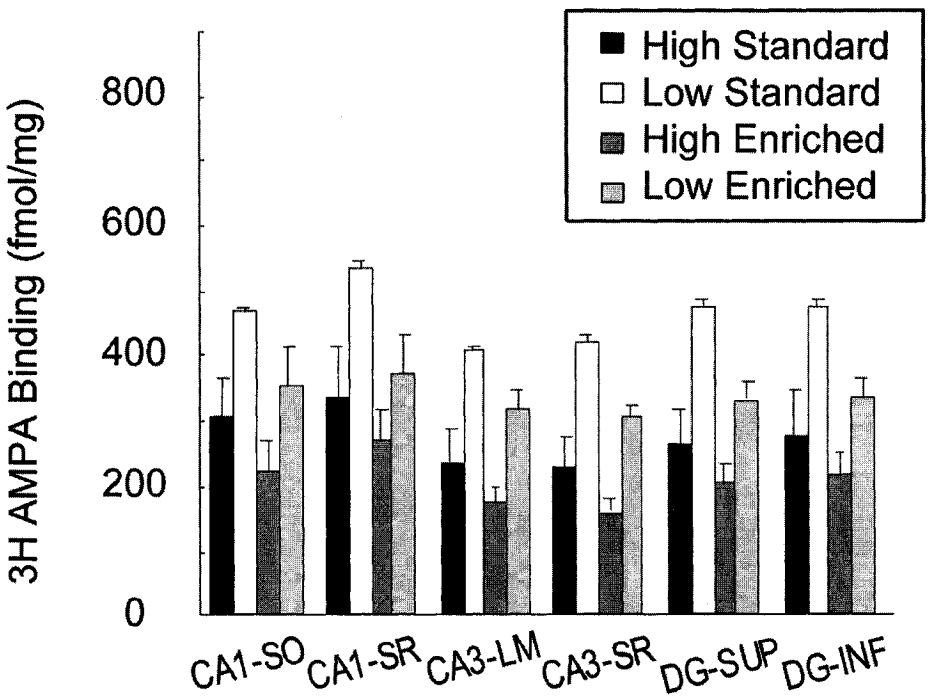


Figure 17. Mean ± SEM hippocampal specific [^{3}H] AMPA binding (n = 4-5/group) for adult offspring of High- and Low-LG mothers reared under standard or enriched housing conditions. (SO- stratum oriens, SR- stratum radiatum, INF- inferior blade, SUP- superior blade) (Bredy et al., 2003).

3.5. DISCUSSION- Maternal care alters hippocampal development and forms a basis for stable, individual differences in learning and memory (Liu et al., 2000). In the present study, we found that post-weaning environmental enrichment completely reversed the effects of maternal care at the level of cognitive performance (i.e. functional reversal). Such effects were apparent on measures of both spatial learning and object recognition where the performance of the enriched offspring of Low-LG mothers was comparable to that of High-LG offspring. Interestingly, there was no significant effect of enrichment on the offspring of High-LG mothers. These findings resemble the results of child intervention programs in humans (Ramey & Ramey 1998a,b). Such programs greatly offset the risk for impaired cognitive development associated with family dysfunction, and such effects are most apparent when development was compromised as a function of early life adversity. Thus, the effects of these "early start" programs are most marked in children from families characterized by turmoil and reduced parental education. In the rat, effects of postnatal forms of "enrichment", such as neonatal handling, are most apparent in animals exposed to prenatal stress (Weinberg et al., 1995; Vallee et al., 1997; Smythe et al., 1994). The common theme is that adversity in early life appears to enhance sensitivity to later forms of enrichment.

The obvious question concerns the issue of mechanism: Does functional reversal through enrichment involve the same neural targets that are sensitive to maternal care? Environmental enrichment did not reverse the effects of maternal care at the level of structure. The offspring of High-LG mothers show increased NMDA receptor binding associated with elevated expression of the mRNAs encoding for the

NR2A and NR2B subunits of the NMDA receptor (Liu et al., 2000). In the adult rat, spatial learning/memory is dependent upon hippocampal integrity (Morris et al., 1982). Moreover, spatial learning is impaired under conditions of NMDA receptor blockade or NR1 subunit knock-out (Quirion et al., 1995; Gage and Bjorklund, 1986; Morris et al., 1986; Bailey et al., 1996; Bliss and Collingridge, 1993). Likewise, hippocampal long-term potentiation, often considered as a model of neuroplasticity associated with learning and memory, is enhanced by over-expression of NMDA receptor subunits at the level of the hippocampus (Tang et al., 1999), and the High-LG offspring showed enhanced long-term potentiation in the dentate gyrus (LTP) (Figure 15). We found that environmental enrichment, despite clear affects on spatial learning/memory, did not reverse the effects of low maternal care on LTP or hippocampal NMDA receptor binding.

These findings suggest that environmental enrichment stimulates the development of neural systems that, ultimately, compensate for the effects of reduced maternal investment (i.e., Low-LG maternal care) during infancy. Rampon et al. (2000) showed an increase in synaptic density in area CA1 after exposure to environmental enrichment in NR1 knockout mice. They suggest that NR1 activity is not specifically required for structural plasticity in the CA1 region of adult animals induced by enrichment. The findings are consistent with the idea that enrichment could "compensate" for the effects of low maternal LG on NMDA receptor systems rather than directly reverse the effects.

Interestingly, environmental enrichment significantly decreased AMPA receptor binding in both groups and reversed the difference in AMPA binding in the

CA1 stratum radiatum and inferior blade of the dentate gyrus in the Low-LG offspring (Figure 17). AMPA receptors play an important role in activity-dependent synaptic plasticity and, like NMDAR, are functionally dependent on their subunit composition (Malinow et al., 2000; Zamanillo et al., 1999; Seifert et al., 2000). The maternal care effect on AMPA binding and the learning/memory deficits is perhaps surprising, but is consistent with previous work demonstrating that among aged rats, those exhibiting more pronounced cognitive impairment have higher AMPA binding in the hippocampus (LeJeune et al., 1996).

Activity-dependent stabilization of synaptic contacts and the refinement of neuronal networks are associated with the delivery of functional AMPA receptors to synapses from non-synaptic sites (the "silent" synapse model; Malenka and Nicholl, 1997; Shi et al., 1999; Malinow et al., 2000). The apparent contradiction here involves the association between enhanced cognitive performance and decreased AMPA binding in the hippocampus. Receptor binding assays, however, provide a functional measure of receptor activity and do not necessarily reflect absolute receptor levels. Factors such as receptor trafficking (Shi et al. 2001) influence the availability of functional membrane receptor sites. At this point these findings are best interpreted as suggesting an effect of maternal of care on AMPA receptor function and a possible reversal with environmental enrichment. The nature of this effect and its relationship to hippocampal function remains to be clarified.

3.6. CONCLUDING REMARKS- The data from this study suggest that environmental enrichment reverses the effect of reduced maternal care on

hippocampal dependent learning. The neural mechanism for the effect of enrichment involves changes in AMPA receptor binding and likely involves compensatory changes that result in improved cognitive performance in tests of spatial and object recognition learning. The two obvious questions here concern the nature of the compensatory changes associated with enrichment as well as the basis for the increased sensitivity to enrichment in animals previously reared under conditions of "impoverishment".

CHAPTER 4- PERIPUBERTAL ENRICHMENT REVERSES THE EFFECTS OF MATERNAL CARE ON HIPPOCAMPAL DEVELOPMENT THROUGH ALTERATIONS OF THE GLUTAMATE RECEPTOR

4. RATIONALE- After considering the data from the chapter 3 and the initial findings by Liu et al (2000), the molecular mechanisms mediating the effect of environmental enrichment were clearly not known, therefore, a more thorough investigation of the underlying mechanisms known to mediate hippocampal synaptic plasticity was required. Thus, the main objective of these experiments was to elucidate the neural mechanisms mediating the effect of maternal care and environmental enrichment on synaptic plasticity in the hippocampus. We examined several putative factors known to contribute to hippocampal function in an attempt to demonstrate how enrichment can rescue the brain from compromised development early in life. It was hypothesized that environmental enrichment would reverse the effect of reduced maternal care on hippocampal synaptic density and gene expression for subunits of the glutamate receptor known to contribute to synaptic communication.

4.1. ABSTRACT- Maternal care in the rat influences the development of cognitive function in the offspring through neural systems known to mediate activity-dependent synaptic plasticity. The offspring of mothers that exhibit increased levels of pup licking/grooming (High-LG mothers) show increased hippocampal N-methyl-D-aspartate (NMDA) subunit mRNA expression, enhanced synaptogenesis, and improved hippocampal-dependent spatial learning by comparison to animals reared

by Low-LG mothers. The effects of maternal care on cognitive function are reversed with peripubertal environmental enrichment, however, the neural mechanisms mediating this effect are not known. In these studies we exposed the offspring of High- and Low-LG mothers to environmental enrichment from days 22-70 of life and measured the expression of genes encoding for glutamate receptor subunits and synaptophysin expression as a measure of synaptic density. Environmental enrichment reversed the effects of maternal care on synaptic density and this effect was, in turn, associated with a reversal of the effect of maternal care on the NR2A and NR2B subunits of the NMDA receptor, as well as effects on AMPA receptor subunits. Finally, direct infusion of an NR2B-specific NMDA receptor antagonist into the hippocampus eliminated the effects of maternal care on spatial learning/memory in the Morris water maze. These findings suggest that 1) the effects of maternal care are mediated by changes in NR2B gene expression and 2) that environmental enrichment reverses the effects of maternal care through the same genomic target, the NR2B gene, and possibly effects on other subunits of the NMDA and AMPA receptors.

4.2. INTRODUCTION- Maternal care in the rat influences the development of the hippocampus and thus spatial learning/memory through neural systems known to mediate activity-dependent synaptic plasticity. The offspring of mothers that exhibit an increased frequency of pup licking/grooming (High-LG mothers) over the first week of life show increased hippocampal synaptogenesis, and enhanced expression of genes encoding for the N-methyl-D-aspartate (NMDA) receptor complex, relative to

the offspring of mothers that exhibit low levels of pup licking/grooming (Liu et al., 2000). Not surprisingly, the offspring of High-LG mothers also show improved performance on hippocampal-dependent tests of learning and memory, such as the Morris water maze (Liu et al., 2000; Bredy et al., 2003). The results of cross-fostering studies provide evidence that the maternal behavior directly affects hippocampal development and cognitive function in the offspring (Liu et al., 2000).

Environmental conditions during the peripubertal period affect brain morphology and behavior over the lifespan (van Praag, Kempermann and Gage, 2000; Rampon et al., 2000 for reviews). Environmental enrichment enhances learning and memory in various behavioral tasks, including spatial memory in the Morris water maze, and recognition memory in the novel object recognition test (Falkenberg et al., 1992; Nilsson et al., 1999; Kempermann, Kuhn and Gage, 1997; Rampon et al., 2000). Further, animals exposed to environmental enrichment show enhanced hippocampal long-term potentiation, and increased expression of brain-derived neurotrophic factor and nerve growth factor, two proteins associated with the maintenance of synaptic contacts (Falkenberg et al., 1992; Pham et al., 1999; Young et al., 1999; Duffy et al., 2001).

Environmental enrichment reverses the effect of prenatal stress on spatial learning, neuronal proliferation and survival, and synaptogenesis (Koo et al. 2003). The detrimental effects of social isolation from postnatal days 22-49 on spatial learning, hippocampal neurogenesis, and synaptic plasticity in area CA1 are reversed by subsequent enriched social housing conditions from postnatal 50-77 (Lu et al., 2003). Environmental enrichment also reverses deficits in hippocampal NR1 mRNA

expression and spatial learning induced by prior exposure to lead (Guilarte et al., 2003). Moreover, environmental enrichment attenuates memory deficits in CA1-specific NMDA receptor knockout mice with an accompanying increase in dendritic remodelling and synaptic density in area CA1 (Rampon et al., 2000). We previously reported that peripubertal environmental enrichment reversed the effect of maternal care on hippocampal-dependent spatial and non-spatial learning (Bredy et al., 2003). As adults, the offspring of Low-LG mothers housed under conditions of enrichment from Days 22-70 of life did not differ from those of High-LG mothers on measures of spatial learning or object recognition memory. Based on a NMDA and AMPA receptor binding study, we suggested that the reversal effect was perhaps due to compensatory changes in the brain occurring as a result of exposure to enrichment (Bredy et al., 2003).

These findings provide compelling evidence that environmental enrichment can reverse cognitive 'deficits' associated with 'insults' or 'deprivation' during early development, however, the mechanism for such effects remain largely unknown. It is not clear, for example, whether the functional reversal reflected in behavioural performance is reflected at the level of changes in gene expression. Does enrichment reverse deficits in spatial learning and memory through effects on hippocampal synaptic plasticity and glutamate receptor subunit expression, or might the effects of enrichment involve compensatory effects exerted at other genomic targets that then mask the persisting deficits? We addressed these questions in the present experiments and found evidence for a reversal of the effects of postnatal maternal care on both function and underlying mechanisms in animals exposed to peripubertal enrichment.

Thus, enrichment reverses the effects of maternal care on NMDA and AMPA receptor subunits, and thus the effects on cognitive performance. Of particular interest are the effects on the NR2B subunit of the NMDA receptor, which has been closely associated with spatial learning/memory.

4.3. Materials and Methods- *Animals-* The mothers were Long-Evans hooded rats born in our colony and derived from females obtained from Charles River Canada (St. Constant, Quebec). Mothers and litters were housed in 46 cm x 18 cm x 30 cm "Plexiglass" cages with food and water provided ad libidum. The colony was maintained on a 12:12 light:dark schedule with lights on at 0800. The animals underwent routine cage maintenance beginning on Day 12, but were otherwise not manipulated. All procedures were performed according to guidelines developed by the Canadian Council on Animal Care with protocols approved by the McGill Committee on Animal Care.

Maternal behavior- Please refer to that described in Chapter 2.

Postweaning housing conditions- Please refer to that described in Chapter 3.

Tissue Preparation- Brains were obtained from adult (approximately 90 days of age) offspring of High- and Low-LG mothers by rapid decapitation, snap frozen in -70°C isopentane and stored at -80°C. For *in situ* hybridization, frozen coronal brain sections (16μm) were cut on a cryostat (male rats, n = 5/group), thaw-mounted on

polylysine-coated slides and stored at -80°C until assay. For western blot, rats (male rats, n=5/group) were sacrificed by rapid decapitation, hippocampi dissected, and stored at -80°C until assay.

Gel Electrophoresis and Western Blot- Protein concentration of fractionated synaptosomal preparations was determined according to the method of Bradford (Bradford, 1976). Synaptophysin protein expression was determined by electrophoresis and western blot analysis using a mouse monoclonal anti-synaptophysin antibody (Sigma, St. Louis, Missouri). Individual samples, each constituting one male rat hippocampus (n = 5/group), were run on a single 15 well gel. Briefly, samples were prepared on ice (to a final volume of 20 µl), vortexed, and denatured for 10 min. at 70°C. Gels were run with 1X TBS-T and the proteins transferred onto PVDF membranes. The membrane was blocked with 5 ml of 5% skim milk powder (Carnation) in TBS-T for 1 h at room temperature, washed with TBS-T for 5 min (3X) and incubated with 5 ml synaptophysin Ab (1:2000) in TBS-T in 0.5% skim milk powder for 24 h at 4°C. The membrane was washed with TBS-T (3X), incubated for 1 h with anti-mouse horseradish peroxidase linked secondary Ab in TBS-T (1:2500), and washed in TBS-T for 10 min. (5X) and 20 min. (1X). Signal detection was performed using an enzyme chemiluminescence kit (ECL, Amersham). Optical density readings of the film were taken using an image analysis system (MCID, St. Catherines, Ontario). The membrane was then stripped and probed with anti-tubulin (Sigma, St. Louis, Missouri) (1:2000) as a control for loading errors. A 2-way ANOVA (maternal care x housing) with relevant posthoc tests (Tukey/Kramer)

was used for analysis, and we used the ratio of the relative optical density for synaptophysin over the relative optical density for tubulin, thus providing an internal control for each individual sample.

In situ Hybridization- In preparation for the hybridization experiments, sections were pre-fixed in a 4% paraformaldehyde solution for 10 min. Sections were then washed in 2XSSC buffer (2 x 5 min.) and in 0.25% acetic anhydride and 0.1 M triethanolamine solution (pH 8.0; 1 x 10 min.). Sections were then dehydrated using a 50-100% ethanol gradient, placed in chloroform for 10 min., following be a rehydration in 95% ethanol. Sections were then incubated overnight at 37^{o}C with 75 µl/section of hybridization buffer containing 50% formamide, 10 mM dithiothreitol, 10 mM Tris (Ph 7.5), 600 mM sodium chloride, 1 mM EDTA, 10 % dextran sulfate, 1 X Denhardt's solution, 100 µg salmon sperm DNA, 100 µ g/ml yeast tRNA, with 1 x 10^{6} CPM [^{35}S] ddATP labelled oligonucleotide probe. Oligonucleotide probes with the following sequences; NR2A-5'-AGAAGGCCCGTGGGGAGCTTTCCCTTTG-GCTAAGTTTC-3' genbank accession no: NM012573 (Monyer et al., 1992), NR2B-5'-GGGCCTCCTGGCTCTCTFCCATCGGCTAGGCACCTGTTGTAACCC-3'gen-bank accession no: NM012574 (Monyer et al., 1992), GluR1-5'-GTCACTG-GTTGTCTGGTCTCGTCCCTCTTCAAACTCTTCGCTGTG-3' genbank accession no: NM031608 (Behnam Ghasemzadehet al., 1999), GluR3- 5'-AGGG-CTTTGTGGGTCACGAGGTTCTTCATTGTTGTCTTCCAAGTG3' genbank acc-ession no: NM032990 (Behnam Ghasemzadehet al., 1999), were synthesized (Beckman 1000 DNA Synthesizer, Beckman, USA) and labelled using a DNA 3'-end

labelling kit (Boehringer Mannheim, USA). Preliminary studies using a scrambled version of the probes yielded no specific signal on brain sections (data not shown). Following hybridization, slides were washed 4 X 30 min. in 1X SSC at 55°C, rinsed briefly in water, dried, and apposed to Hyperfilm for 2-3 weeks. The hybridization signal within various brain regions was quantified using densitometry with an image analysis system (MCID, St. Catherines, Ontario). The data are presented as relative optical density (ROD) units following correction for background and analysed by 4-way ANOVA (maternal care x housing x region x hemisphere). The anatomical level of analysis was verified with Nissl-staining of sections following autoradiography.

Surgery and Cannulae placement- Rats were anaesthetised (Somnitol, 65mg/kg, i.p.), bilateral cannulae were inserted using stereotaxic techniques under aseptic conditions, and rats were given a minimum of 5 days to recover before behavioral testing. Coordinates used (relative to bregma) were; anteroposterior, 3.8 mm; mediolateral, ±3.1 mm; and dorsoventral, along a 0.5 mm track from 2.1 mm from the surface of the brain. Vehicle (0.1% DMSO) or drug (Ifenprodil, 5nmol/ul) was infused over 1 min., approximately 10 min. prior to exposure to the behavioral task. Injection cannulae were left in place for an additional 1 min. after injection and were "capped" with a 1µl air bubble by injecting the air as the cannula was slowly removed. Upon completion of the experiment, rats were sacrificed by rapid decapitation, cannulae removed, and brains were cut in 40µm sections, counterstained with cresyl violet and cannulae placement confirmed (Figure 18).

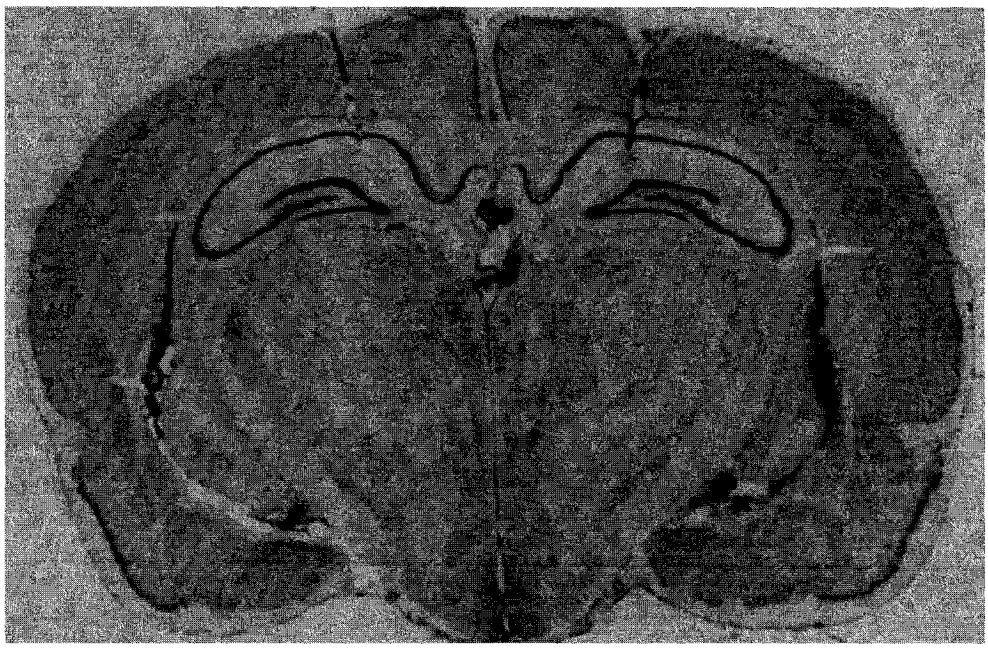


Figure 18. Representative photomicrograph of cannulae placement in area CA1

Morris Water Maze- 10 min. prior to exposure to the apparatus, rats were given a single bilateral intra-hippocampal infusion of the NR2B antagonist Ifenprodil (5nmol/1μl, Sigma, St. Louis, MO) or vehicle (0.1% DMSO). The concentration of drug antagonist was based on pilot studies using uncharacterised animals, where we determined that a 1nmol/1μl was insufficient, and 5nmol/1μl sufficient, to impair acquisition of the Morris water maze task. At the time of our experiment, there was no literature on the effects of intra-hippocampal infusion of iIfenprodil on spatial learning; therefore, we approximated our concentration based on *in vitro* work demonstrating the binding affinity of Ifenprodil to glutamate receptors expressing the NR2B subunit (Grimwood et al., 2000). Rats were required to locate a submerged platform (15 x 15 cm) that was 1 cm below the water line in a 1.5 m-diameter pool using only ambient spatial cues available within the testing room (Morris, 1982). The water was made opaque by a layer of white polypropylene pellets that floated on the surface. Between 1000 and 1600, the rats were given 10 trials with reversal of platform location on trial 6 (minimum 20 minutes inter-trial interval) with the platform submerged. On day 2, rats were given a probe trial (60 s) in which the platform was removed to determine spatial bias for platform location during reversal training on the previous day. Pathlength (cm) and time spent in quadrant (s) were recorded using the HVS video tracking system (San Diego Instruments). Pathlength was analysed by between-within ANOVA (maternal care x treatment x time) and quadrant dwell time (s) was analysed by 2 way ANOVA (maternal care x treatment) and Tukey/Kramer posthoc tests were performed on individual trials.

4.4. RESULTS- *Synaptic density.* There was a significant main effect ($F_{1,16} = 22.0$, $p < .001$) of housing condition on synaptophysin protein expression (Figure 2). Enriched animals showed significantly higher synaptophysin levels than standard housed animals. Importantly, there was a significant maternal care x housing interaction ($F_{1,16} = 5.26$, $p < .05$) with the enriched offspring of Low-LG mothers showing a greater increase in synaptophysin than the enriched offspring of High-LG mothers. Similar to the previous report by Liu et al (2000), post-hoc analysis revealed that under standard housing conditions, the offspring of High-LG mothers showed significantly higher levels synaptophysin expression than the offspring of Low-LG mothers ($p < .05$).

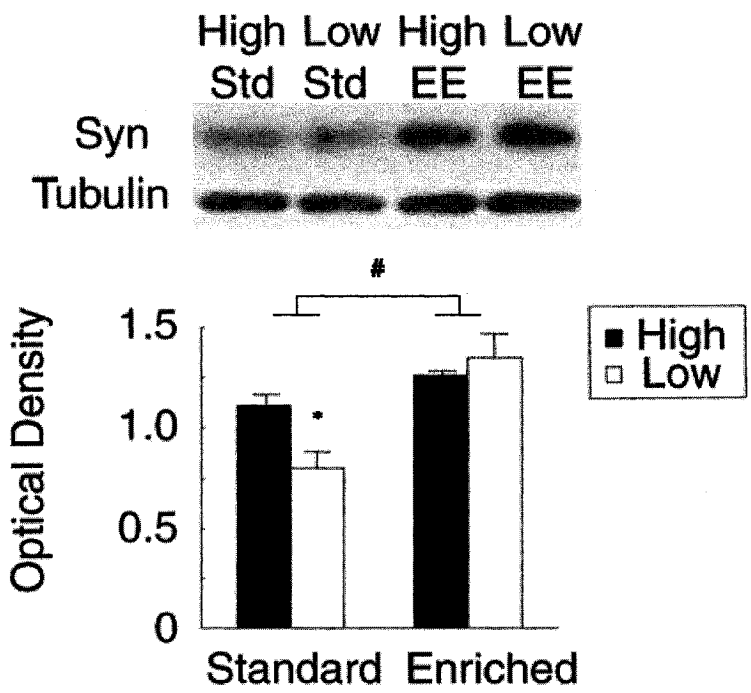


Figure 19. Mean ±SEM whole hippocampal synaptosomal preparation levels of synaptophysin protein expression (n = 5/group) in the offspring of high- and low-LG mothers reared under standard or enriched housing conditions, expressed as a ratio of relative optical density units. Upper panel shows a representative Western blot with α-tubulin used to control for loading errors (* $p < .05$, # $p < .01$).

NMDA receptor subunit NR2A mRNA expression. There was a significant main effect ($F_{1,72} = 14.25$, $p < .001$) of maternal care on NR2A mRNA expression as, overall, the offspring of Low-LG mothers showed reduced NR2A mRNA expression (Figure 3). There was a significant main effect of region ($F_{1,72} = 126.41$, $p < .0001$) on NR2A mRNA expression, as NR2A mRNA levels were lower in area CA1 than in the DG, however, there was no effect of laterality nor any interaction involving laterality. There was a significant main effect ($F_{1,72} = 20.54$, $p < .0001$) of housing condition on NR2A mRNA expression; enriched animals showed higher levels than standard housed animals. There was a significant maternal care X housing interaction ($F_{1, 72} = 8.40$, $p < .01$) on NR2A mRNA expression, as the offspring of Low-LG mothers raised under conditions of enrichment showed higher NR2A mRNA expression than the offspring of Low-LG mothers raised under standard housing conditions.($p < .05$)

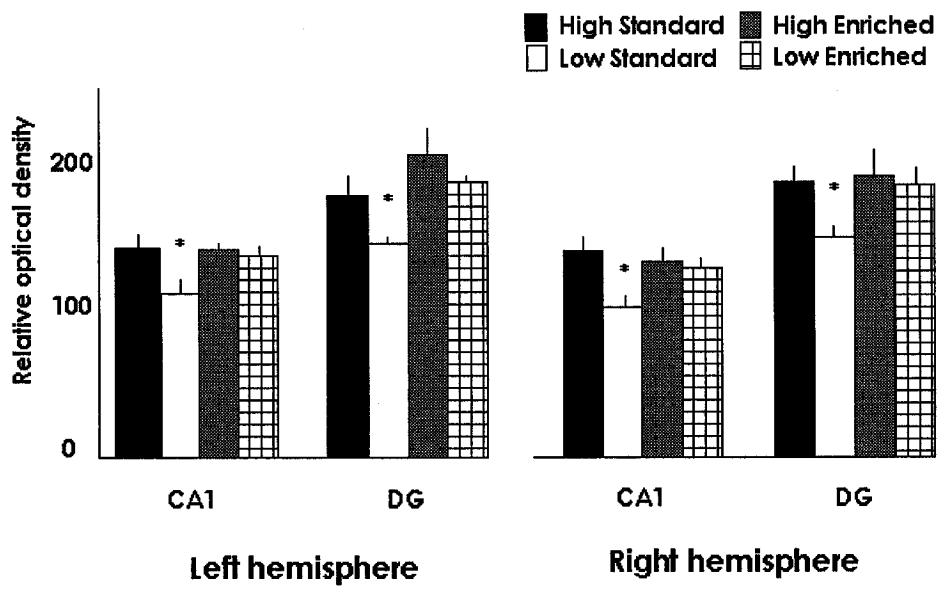
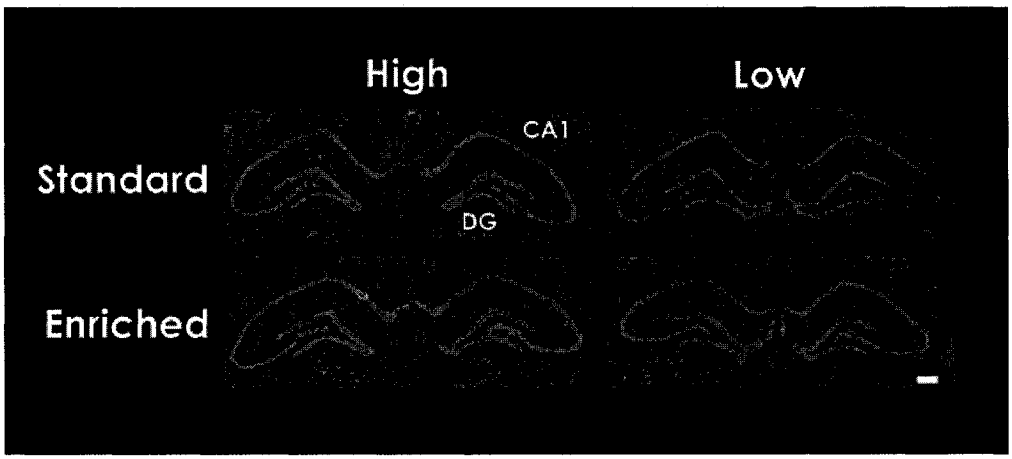


Figure 20. Mean ±SEM hippocampal NR2A mRNA expression (n = 5/group) in the offspring of high- and low-LG mothers reared under standard or enriched housing conditions, expressed as a ratio of relative optical density units (* $p < .05$).

NMDA receptor subunit NR2B mRNA expression. There was a significant main effect ($F_{1,72} = 12.17$, $p < .001$) of maternal care on NR2B mRNA expression as the offspring of Low-LG mothers showed reduced NR2B mRNA expression compared to the offspring of High-LG mothers (Figure 4). There was a significant main effect ($F_{1,72} = 71.24$, $p < .0001$) of region on NR2B mRNA expression, as NR2B mRNA expression was lower in area CA1 and than in the DG, with no main or interaction effects involving laterality. There was a significant main effect ($F_{1,72} = 4.65$, $p < .05$) of housing condition on NR2B mRNA expression, as enriched animals showed higher levels than standard housed animals. There was a significant maternal care X housing X region interaction ($F_{1, 72} = 4.09$, $p < .05$) on NR2B mRNA expression as the offspring of Low-LG mothers raised under conditions of enrichment showed higher NR2B mRNA expression in area CA1 than the offspring of Low-LG mothers raised under standard housing conditions. There were no effects of maternal care or housing on NR2B mRNA levels in the DG.



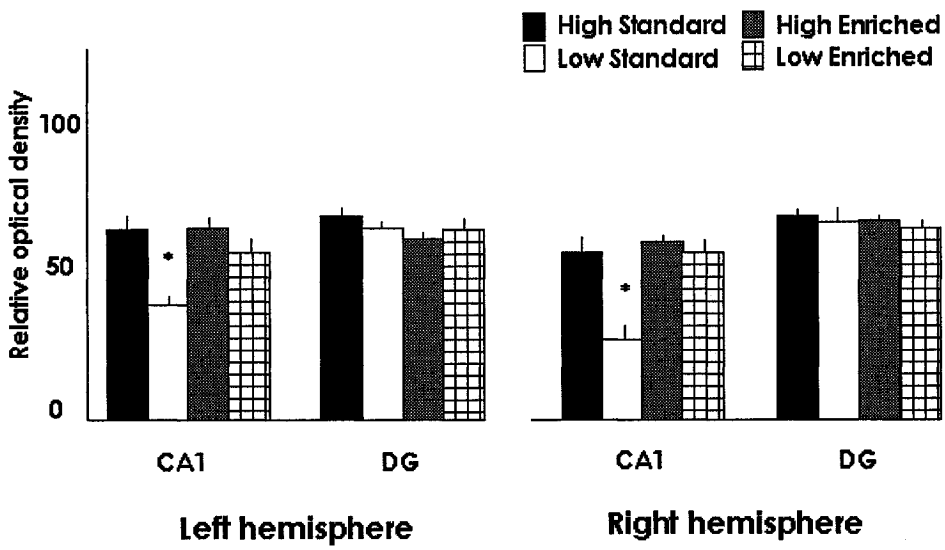


Figure 21. Mean ±SEM hippocampal NR2B mRNA expression (n = 5/group) in the offspring of high- and low-LG mothers reared under standard or enriched housing conditions, expressed as a ratio of relative optical density units (* $p < .05$). Upper panel shows a representative colour enhanced photomicrograph of hippocampal NR2B mRNA expression (scale bar: 10μm).

AMPA receptor subunit GluR1 mRNA expression. There was a significant interaction effect ($F_{1, 72}$= 14.21, $p < .001$) between hemisphere and region on GluR1 mRNA expression as in the right hemisphere, as GluR1 mRNA expression was lower in area CA1 than in the DG (Figure 4). There was a significant interaction between hemisphere and housing ($F_{1, 72}$= 14.88, $p < .001$) on GluR1 mRNA expression as in the right, but not the left hemisphere, enriched animals showed higher GluR1 mRNA levels than standard housed animals. There was a significant interaction between hemisphere and maternal care ($F_{1, 72}$= 14.21, $p < .001$) on GluR1 mRNA expression as in the right, but not the left hemisphere, the offspring of High-LG mothers showed higher GluR1 mRNA expression and this effect was most pronounced in standard housed animals. In the left hemisphere, simple effects analysis revealed a significant main effect of housing ($F_{1, 36}$= 6.95, $p < .01$) and region ($F_{1, 36}$= 6.52, $p < .05$) on GluR1 mRNA expression as GluR1 mRNA expression was highest in standard housed animals and the dentate gyrus showed the highest GluR1 mRNA expression. In the right hemisphere, there was a significant maternal care X housing interaction effect ($F_{1, 36}$= 3.94, $p < .05$) as under standard housing conditions, the offspring of High-LG mothers showed significantly ($p<.05$) increased GluR1 mRNA expression, a difference that was eliminated in animals housed under conditions of enrichment.

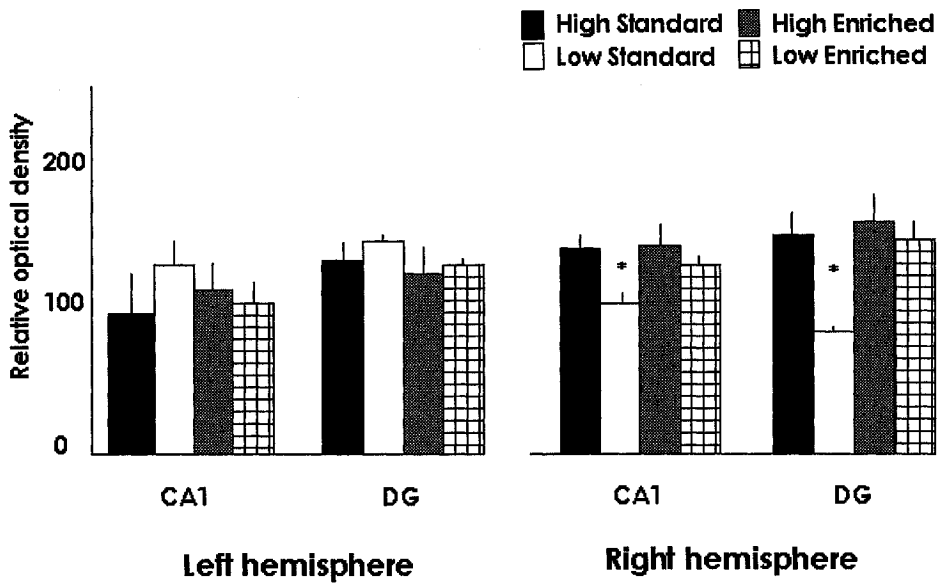


Figure 22. Mean ±SEM hippocampal GluR1 mRNA expression (n = 5/group) in the offspring of high- and low-LG mothers reared under standard or enriched housing conditions, expressed as a ratio of relative optical density units (* $p < .01$).

AMPA receptor subunit GluR3 mRNA expression. There was a significant interaction between hemisphere and housing ($F_{1, 72}$= 9.58, $p < .01$) on GluR3 mRNA expression as in the right hemisphere, as enriched animals showed higher GluR3 mRNA levels than standard housed animals (Figure 5). Tukey/Kramer post-hoc analysis revealed that the effect was most pronounced in area CA1 where under standard housing conditions, the offspring of Low-LG mothers showed reduced expression of GluR3 mRNA compared to the offspring of High-LG mothers ($p < .05$), a difference that was eliminated under conditions of enrichment. In the left hemisphere, simple effects analysis revealed a significant main effect of housing ($F_{1, 36}$= 10.32, $p < .01$) on GluR3 mRNA expression as standard housed animals showed higher GluR3 mRNA expression than enriched animals.

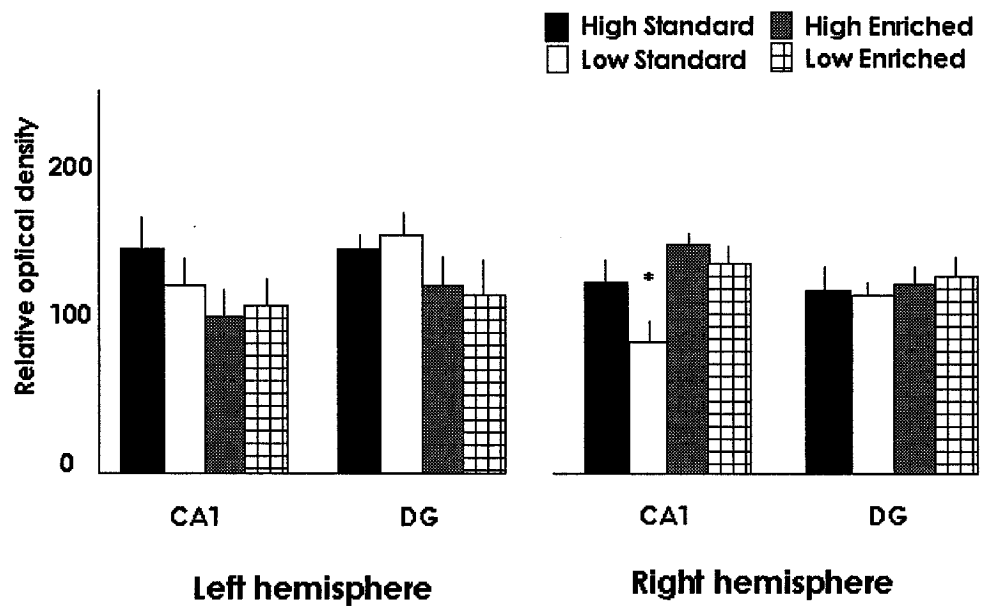


Figure 23. Mean ±SEM hippocampal GluR3 mRNA expression (n = 5/group) in the offspring of high- and low-LG mothers reared under standard or enriched housing conditions, expressed as a ratio of relative optical density units (* $p < .05$).

Morris Water Maze. Five animals were removed from the analysis due to poor cannulae placement. Overall, there were significant main effects of maternal care ($F_{1,31} = 4.54$, $p < .05$), treatment ($F_{1,31} = 5.56$, $p < .05$) and trial ($F_{9,279} = 15.40$, $p < .0001$) on pathlength (see Figure 7). The offspring of High-LG mothers showed significantly shorter pathlength than the offspring of Low-LG mothers and Ifenprodil-treated animals showed significantly longer pathlength than vehicle-treated controls. Over trials, pathlength decreased for all animals. Post-hoc analysis (Tukey/Kraemer test) showed that in the standard housed group, the vehicle treated offspring of High-LG mothers had shorter pathlength than all other groups on trials 4, 5, and 6 (all p's < .05), while Ifenprodil-treated offspring of High-LG mothers did not differ from vehicle treated Low-LG offspring. There was no significant effect of maternal care or treatment on time spent in the correct quadrant during the probe on day 2 (data not shown).

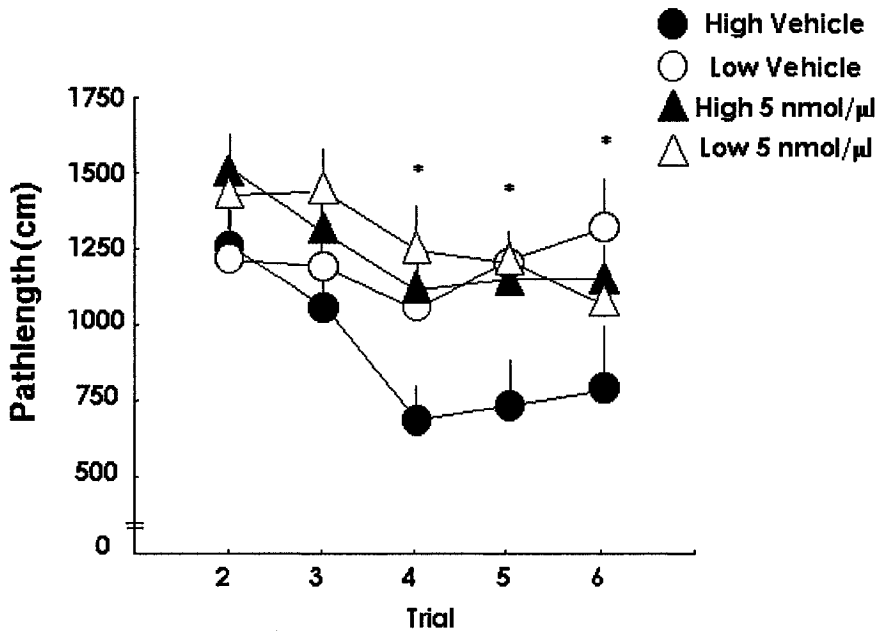


Figure 24. Mean ±SEM pathlength (cm) (n = 8-10/ group) from trials 2-6 of Morris water maze training for offspring of high- and low-LG mothers treated with either vehicle (0.1% DMSO) or the NR2B specific antagonist Ifenprodil (5 nmol/1µl) (* $p < .05$).

4.5. DISCUSSION- In the study presented in chapter 3, we found that environmental enrichment reversed the effects of maternal care on spatial learning/memory performance in the Morris water maze. The results of the present study show that peripubertal environmental enrichment completely reversed the effects of maternal care on hippocampal synaptophysin expression, and the expression of NMDA and AMPA mRNA subunits known to contribute to hippocampal-dependent learning and memory. As in the previous study (Bredy et al. 2003), we found no significant effect of enrichment on the offspring of High-LG mothers.

Cooper and Zubek (1958) introduced the possibility that environmentally-induced and/or inherited behavioural traits could be reversed with enrichment, and thus there exist individual differences in sensitivity to the effects of environmental enrichment. Among animals selectively bred for performance in the Hebb-Williams maze, enrichment during the peripubertal period improved performance of 'maze-dull' rats, with no effect on 'maze bright' rats (Cooper and Zubek, 1958). Likewise, among Roman high- and low-avoidance (RHA/Verh and RLA/Verh) rats, selectively bred for rapid (RHA/Verh) versus poor (RLA/Verh) acquisition of avoidance learning the effects of environmental enrichment on learning and memory are apparent in the RLA/Verh line, with little or no effect in the RHA/Verh line (Fernandez-Teruel et al., 1997; Fernandez-Teruel et al. 2002). Similarly, the effects of a wide variety of perinatal insults or environmental deprivation on spatial learning/memory, neuronal proliferation and survival, and synaptic density are reversed by peripubertal enrichment, and in each case the effects of enrichment are greater among perinatal treatment groups by comparison to controls (Lee and Rabe, 1999; O'Leary et al.,

2002, Guilarte et al., 2003; Koo et al. 2003; Lu et al. 2003). These findings clearly reflect the sustained plasticity within the hippocampus, and the capacity for the reversal of previously established 'deficits' in synaptic density and cognitive performance through environmental enrichment at later stages of development.

The offspring of Low-LG mothers are sensitive to the effects of environmental enrichment, while the offspring of High-LG mothers show little or no response to enrichment. This difference in individual sensitivity is apparent at multiple levels of analysis and is consistent with studies that demonstrate the influence of factors at one stage development influencing responsivity to factors at later stages of development (i.e. Cooper and Zubek, 1957). A central question that comes to mind is the specificity of these effects: are the Low-LG offspring simply more sensitive to all forms of stimulation or are the High-LG offspring less adaptive and less sensitive to all forms of stimulation? One way to address this issue is to demonstrate that under certain conditions, the Low-LG offspring have an advantage while the High-LG offspring are compromised in some way. In contextual fear conditioning, a task that relies heavily on the amygdala, this is exactly the case. The Low-LG offspring clearly show enhanced learning by comparison with High-LG offspring (R. Bagot, unpublished observations). The difference in fear conditioning is reflected by differences in subunit composition of the GABA(A) receptor, with the High-LG offspring showing more $\gamma 2$ mRNA and protein expression in the basolateral amygdala (Caldji et al., 2004).

Given that the High-LG offspring show enhanced learning in a variety of tasks, it is unlikely that they are less adaptive or less sensitive to environmental factors. The data would suggest that they are more resilient and assume a more robust phenotype that is tailored for a stable environment. On the other hand, the Low-LG offspring may be the optimal phenotype. Under stable environmental conditions Low-LG offspring appear to be impaired, however, they respond well to enrichment and other forms of stimulation suggesting that they assume a more adaptive phenotype perfectly suited for living in a dynamic, ever changing environment, akin to the real world rather than the safe confines of the laboratory setting.

Synaptic plasticity is associated with learning, and rats with spatial learning deficits have a significant reduction in synaptophysin expression (Van Reempts et al., 1992; Moser et al, 1994; Smith et al., 2000). Environmental enrichment increases hippocampal synaptic vesicle density, synaptophysin expression, and enhances hippocampal synaptic plasticity (Mollgaard et al., 1971; Saito et al., 1994; Nakamura et al., 1999; Foster et al., 2000). The offspring of Low-LG mothers show decreased hippocampal synaptophysin expression by comparison with the offspring of High-LG mothers, a difference that is eliminated after exposure to environmental enrichment (Figure 19). These findings are consistent with the idea that the effects of environmental enrichment on cognitive performance are accompanied by enhanced synaptic density. The critical question concerns the molecular mechanisms underlying the effects of environmental enrichment.

Peripubertal environmental enrichment during adolescence reversed the effect of maternal care on genes encoding for glutamate receptor subunits known to

contribute to hippocampal-dependent learning and synaptic plasticity. Enrichment reversed the effect of maternal care on the expression of the NR2A and NR2B subunits of the NMDA receptor complex in area CA1 of the hippocampus (Figure 20 and 21), as well as the effects on GluR1 and GluR3 mRNA expression in the right hippocampal area CA1 (Figure 22 and 23). Importantly these effects parallel those of enrichment on both synaptic density and spatial learning/memory. Thus, in each case the effect of enrichment on glutamate receptor subunit expression was specific to the offspring of Low-LG mothers, with no apparent effect on the offspring of High-LG mothers.

The NMDA receptor complex, and the NR2B subunit in particular, is interesting because of its importance in synaptic plasticity and hippocampal-dependent learning/memory (Tang et al, 1999). The adult offspring of High LG mothers show increased NR2B mRNA expression (Liu et al., 2000 and Figure 3). Importantly, the effect of maternal care on spatial learning/memory is eliminated with intra-hippocampal infusion of the NR2B-specific, NMDA receptor antagonist, Ifenprodil. These findings suggest that increased levels of pup licking/grooming over the first week of life enhance the expression of NR2B expression in the CA1 region of the hippocampus, resulting in improved performance in the Morris water maze. The effect of environmental enrichment at later stages in development is to reverse the group differences in NR2B expression, and thus eliminate the effect of maternal care on hippocampal-dependent learning/memory. This conclusion is consistent with the finding of Lee et al. (2003) showing that effects of environmental enrichment on spatial learning/memory are blocked by Ifenprodil, as well as with those of studies

with transgenic animals. Transgenic mice over-expressing the NR2B subunit exhibit enhanced hippocampal LTP and improved learning and memory compared to wild-type controls (Tang et al., 1999). After exposure to environmental enrichment, wild-type mice showed an overall improvement in contextual and cued conditioning, fear extinction and novel object recognition learning, with little or no effect of enrichment on the performance of the NR2B transgenic mice (Tang et al. 2001). These findings directly parallel those of the current study with offspring of High (increased NR2B expression) and Low LG mothers.

In the Tang et al. (2001) study, enrichment was also associated with an increase in the expression of the NR2A, NR2B, and GluR1 subunits in both wild-type controls and NR2B transgenics. These findings suggest that environmental enrichment might enhance learning/memory through effects on the expression of multiple glutamate receptor subunits in the hippocampus. In the current study we found effects of enrichment on NR2A, GluR1 and Glur3 expression in the hippocampus. Interestingly, the effects of enrichment on GluR1 and GluR3 expression were unique to the right hippocampus. This finding is comparable to those of Tang and colleagues who found that exposure to novelty during postnatal life enhanced hippocampal volume (Verstynen et al. 2001) and LTP (Zou & Tang 2001), but only in the right CA1 cell field of the hippocampus.

The issue of lateralization and brain asymmetry have been documented in the human literature for many years and recently has emerged in rodent and avian studies (Geschwind and Galaburda, 1987; Tang and Reeb, 2003; Kahn and Bingman, 2004). In early studies, Denenberg (1978) showed that right hemisphere lesion produced a

greater effect than a left hemisphere lesion in rats that received early life stimulation. These initial findings have now been confirmed and extended to the right hippocampus, where early experience leads to a right shift in hippocampal plasticity and function (Tang and Zhou, 2002; Tang and Verstynen, 2002; Tang, 2003). It has been suggested that in birds, the right hemisphere participates in the memory of global, distally located spatial cues, while the left hemisphere participates more in the memory of proximal cues used to locate a goal in space (Tommasi and Vallortigara, 2001). Interestingly, in humans, the right hippocampus shows a temporal gradient of activity during the recall of remote autobiographical memories (Maguire and Firth, 2003). Together, the data clearly suggest the need to consider lateral asymmetry of the hippocampal formation in studies of the contribution of the hippocampus for certain forms of learning and memory.

In the current study, the reasons for such selectivity are not clear, but there is evidence for the potential implications for cognitive function of increased AMPA sensitivity, in part, through effects on NMDA-mediated events. In addition, Lee et al (2003) found that environmental enrichment-induced increases in SGK (a serum and glucocorticoid-inducible kinase implicated in memory consolidation of spatial learning; Tsai et al, 2002) are mediated by the activity of AMPA receptors in area CA1 of the hippocampal formation. The influence of environmental enrichment on hippocampal-dependent learning may therefore be mediated by effects at multiple genomic targets. First, is an NMDA pathway, changes in which are involved in facilitating synaptic communication during the acquisition of novel information. This idea is supported by literature suggesting that the NMDAR is a coincidence detector,

primarily involved in reaction and learning in response to novelty (Wittenberg and Tsien, 2002; Quinlan et al., 2004). Second, AMPARs appear to be involved in the consolidation of spatial memory, through their interaction with SGK and the glucocorticoid-sensitive pathway (Rozendaal, 2000; Tsai et al., 2002; Lee et al., 2003). Indeed, the co-ordinated effects of enrichment-induced changes in NMDA and AMPA receptor function may operate through common intracellular mediators, such as NGFI-A or Arc both of which are expressed in conjunction with stimuli that induce long-term potentiation in hippocampal formation (Worley et al., 1993; Herms et al., 1994), is dependent on the activation of the NMDAR (Cole et al., 1989; Wisden et al., 1990) and is correlated with spatial learning performance (Yau et al., 1996; Guzowski et al., 2000, 2001; Kelly and Deadwyler, 2002, 2003). Further, NGFI-A binding sites have been located on genes encoding synaptophysin (Thiel et al., 1994) and both NGFI-A and Arc are associated with synaptic expansion (Husi et al., 2000). Thus, NGFI-A is in a convenient position to link experience with changes in gene expression and synaptic plasticity associated with learning and memory

A recurring question throughout the studies performed in this thesis concerns the basis for the increased sensitivity to enrichment in animals previously reared under conditions of "impoverishment". Recall the finding in chapter 2; at the time of weaning there was significant increase in GFAP expression in the day 21 offspring of Low-LG mothers (Figure 12). As previously mentioned, the location and nature of the staining was consistent with an enhanced number of mature astrocytes. Perhaps this increase in astrocyte expression is associated with the observed increased sensitivity to environmental enrichment in the Low-LG offspring. Recall that, along with the

changes in glutamate receptor mRNA expression, environmental enrichment imposed between days 22 and 70 of life completely reverses the effect of reduced maternal LG on spatial learning and memory and synaptic density in the offspring of Low-LG mothers, but has no appreciable effect on the offspring of High-LG mothers. Recent reports suggest that astrocytes may actually serve as neuronal precursors and actively promote synaptic maintenance and development (Seri et al., 2001; Ullian et al. 2001). Thus, an increased population of astrocytes in the offspring of Low-LG mothers at the time of weaning could provide a basis for a more substantial neurogenic and/or synaptogenic response to environmental enrichment during the postweaning period.

4.6. CONCLUDING REMARKS- In summary, peripubertal environmental enrichment reverses the effects of maternal care on glutamate receptor subunit expression, synaptic density, and spatial learning/memory. These findings reveal the impressive capacity for plasticity at the level of function, and suggest that enrichment can reverse prior developmental effects at the level of underlying molecular mechanism.

CHAPTER 5- EFFECT OF NEONATAL HANDLING AND PATERNAL CARE ON OFFSPRING COGNITIVE DEVELOPMENT IN THE MONOGAMOUS CALIGORNIA MOUSE (*PEROMYSCUS CALIFORNICUS*)

5. RATIONALE- The main of objective of these experiments was to extend the maternal care concept and begin developing a model where the influence of paternal care on cognitive development in the offspring could be examined. We examined the influence of neonatal handling and paternal care on spatial and non-spatial learning in adult male and female offspring to determine whether there were gender differences in response to paternal care. (Portions of these data have been published in the journal, Hormones and Behavior, vol. 45, pp. 45-54. With the exception of the handling paradigm, I performed all of the experiments and analysis of the data presented in this chapter).

5.1. ABSTRACT- In the laboratory rat and mouse, neonatal handling enhances hippocampal-dependent learning in adulthood, an effect mediated by changes in maternal behaviour towards the handled young. In the present study, we examined the interaction between neonatal handling and biparental care during the early postnatal period and its effect on cognitive function in adult California mice (*Peromyscus californicus*). We characterized the parental behaviour of handled and non-handled father-present and father-absent families over the first 15 days of life. We then assessed cognitive performance of male and female offspring in the Barnes maze and object recognition test after they were 60 days of age. We found that the amount of licking and grooming received by pups was decreased in father-absent families. By

postnatal days 12-15, licking and grooming in handled, father-absent families were equivalent to that of non-handled, father-present families. Handling enhanced novel object recognition in father-present male mice with no effect in females. In the non-handled group, the presence of the father had no effect on object recognition learning in male or female mice. Handling also enhanced spatial learning in the Barnes maze. In non-handled families, the presence of the father appeared to have no effect on spatial learning in the male offspring. Interestingly, spatial learning in non-handled, father-absent, female offspring was similar to that of handled animals. The average amount of licking and grooming received by pups was negatively correlated with the average number of errors made on the first day of reversal training in the Barnes maze. These data support previous findings that neonatal handling facilitates learning and memory in adulthood, suggest that under certain environmental conditions there is a sex difference in the response of pups to paternal care, and further demonstrate the importance of active parental investment for offspring cognitive development.

5.2. INTRODUCTION- The effects of early-life experience such as neonatal handling, and maternal deprivation on the developing hippocampus may be responsible for individual differences in cognitive function in adulthood. For example, neonatal handling enhances cognition and delays age-related learning impairments, while maternal deprivation in infancy diminishes cognitive functioning and exacerbates age-related learning impairments (Meaney et al., 1988; Meaney et al., 1991; Oitzl et al., 2000; Tang, 2001). The effects of early handling and maternal deprivation on behavioural development are mediated by changes in maternal

behaviour towards the young (Levine, 1967; Smotherman, Brown and Levine, 1977; Villescas et al., 1977; Liu et al., 1997; D'Amato et al., 1998). Tactile stimulation derived from maternal licking and grooming of the young appears to be a critical factor for hippocampal development. In rats, the offspring of mothers that show increased licking and grooming exhibit enhanced spatial and non-spatial learning and memory (Liu et al. 2000; Bredy et al., 2003). Further, individual variation in maternal care contributes to differences in cognitive development in humans and primates, as well as rodents (Ruddy and Bornstein, 1982; Bornstein, 1985; Tamaroff et al., 1986; Zaharia et al., 1996; Liu et al., 2000).

While much is known about the influence of maternal care on pup development, very little is known about the contribution of paternal care toward offspring neural and behavioural development. The California mouse (*Peromyscus californicus*) is a biparental rodent that is well suited for the study of the effects of biparental care on offspring development. *P. californicus* is exclusively monogamous in the wild, has persistent pair bonding, and a high level of paternal investment (Dudley, 1974b; Gubernick and Alberts, 1987). Further, male *P. californicus* display all the components of parental behaviour shown by mothers (huddling, licking and grooming, carrying and retrieving), with the exception of arched-back nursing and lactation (Dudley, 1974b; Gubernick and Alberts, 1987). In the wild, the presence of the male is crucial for offspring survival, and in the laboratory, the presence of the male enhances reproductive success and offspring survival when parents are required to forage for food (Gubernick et al., 1993; Cantoni and Brown, 1997; Gubernick and Teferi, 2000; McInroy et al., 2000; Wright and Brown, 2002). Moreover, *P.*

californicus pups reared with both parents receive almost twice as much parental licking and grooming as pups reared with the mother alone (Wright and Brown 2002). The evidence suggests that paternal investment is a direct process whereby the male actively contributes to offspring viability (Cantoni and Brown, 1997; Gubernick and Teferi, 2000; Wright and Brown, 2002).

Over the past 40 years, Levines' (1957) neonatal handling model has provided much information on the effects of early environmental experience on neural development. Confirmation that the effects of handling are mediated, in part, by changes in maternal care has led to a greater understanding of the importance of the mother-infant interaction early in life. With this knowledge of early experience and maternal care effects in mind, we have begun to explore the importance of biparental care for offspring development. There are several reasons for returning to the handling model and combining it with the biparental care concept; 1) until now, the importance of paternal care for offspring development has received little attention since it cannot be examined in the rat, 2) there is little information on the direct effects of handling on cognitive development in both males and females, hence, our aim was to extend the handling literature to include a new model species, *P. californicus*, and 3) by using a manipulation that is known to increase maternal behaviour towards the young, we sought to extend the literature to include the effect of increased biparental care (as well as the effect of handling induced increases in maternal care in the absence of the father), eventually moving towards elucidating and confirming the neural mechanisms mediating the influence of biparental care on offspring brain development.

Maternal licking and grooming contributes to offspring cognitive development in rats and mice (Zaharia et al., 1996; Liu et al., 2000; Bredy et al., 2003), and *P. californicus* pups receive additional licking and grooming from the father (Wright and Brown, 2002). Therefore, this study was designed to examine the interactions between neonatal handling, and biparental behaviour during the early postnatal period and their effects on cognitive function in adult *P. californicus*. We characterized the parental behaviour of handled and non-handled biparental and father-absent family units over days 3-15 of life. We then assessed cognitive performance of male and female offspring in the Barnes maze and object recognition test after they were 60 days of age. We chose the Barnes maze because it is hippocampal-dependent and, while providing similar information about spatial learning, is a less stressful alternative to the Morris water maze (McLay et al., 1999; Poe et al., 2000). We used the object recognition test, although there is some controversy as to whether this task is hippocampal-dependent, because it is a non-spatial, associative memory task that relies heavily on an animals' initial reactivity to novelty (Renner et al., 1992; Rampon et al., 2000b; Mumby et al., 2002). These tests were used because, as previously mentioned, there is little information on the effect of neonatal handling on spatial and non-spatial learning and memory in rodents.

In the present study, we addressed the following questions: (1) What is the effect of neonatal handling on parental care in *P. californicus*? We expected that neonatal handling would increase parental licking and grooming towards the young from birth to weaning; (2) What is the effect of neonatal handling and paternal care on offspring spatial and non-spatial learning and memory in adulthood? If paternal be

haviour contributes to cognitive development, then pups reared without their father will be licked and groomed less often in infancy and exhibit poorer learning and memory in adulthood; and (3) Are there gender differences in offspring cognitive development in response to handling and paternal care? We hypothesized that male and female *P. californicus* would show a comparable response to handling and/or the absence of paternal care, given that in rats postnatal handling delays age-related learning impairment in both sexes (Meaney et al., 1988; Meaney et al., 1991).

5.3. MATERIALS AND METHODS- *Postnatal Environment (Handling paradigm).* Forty *P. californicus* breeding pairs were established in our colony at Dalhousie University. The mice were housed in standard Plexiglas cages with pine bedding, on a 16:8 reversed L: D cycle with lights off at 0900, at a temperature of 22°C, and fed laboratory rodent chow (Agribrand #5001) and water *ad libitum.* At birth, litters (2-3 pups/litter) were randomly assigned to one of four groups with 10 litters/group (non-handled father-present, handled father-present, non-handled father-absent and handled father-absent). The handling procedure was conducted daily from days 1-21 post-parturition (according to Zaharia et al., 1995). Depending on the treatment group, dams or both dams and their mates were removed from the home cage and placed in a holding cage. Pups were then placed into a new cage, with fresh bedding, for 15 min., after which, they were returned to their respective home cage and reintroduced to the parent(s). For the non-handled groups, cages were left undisturbed until weaning at postnatal day 30. In the father-absent groups, males were removed on postnatal day 3. Female California mice have postpartum oestrous and mate within

3 days after parturition. Thus, males were left with females for 3 days after parturition so that females in all groups were pregnant during the study. All mice were weaned at postnatal day 30 and housed in same-sex pairs until testing.

Characterizing Parental Care. We assessed the parental behaviour of 28 litters (n = 7 litters/ treatment group). From postnatal days 3-15, parental licking/grooming of the pups was observed four times per day (0630, 0930, 1300, 1700) with 3 periods during the dark phase and 1 in the light phase. Within each observation period, the parental licking/grooming of the pups was scored every 3 minutes for 1 hour (20 observations/period x 4 periods/day = 80 observations/day). Since we began our observations on postnatal day 3 (when the father was removed), we decided to pool the data into 3-day blocks across the 12-day period of parental observations. The percentage of observation periods with licking and grooming was calculated for each 3-day block, for each group, and analysed by 2 (handling) x 2 (parental care) x 4 (day block) repeated measures ANOVA.

Adult Offspring Cognitive Function

Subjects. Subjects were 78 adult California mice (8-10 males and 8-10 females per group) assigned to each of the four treatment groups. During behavioural testing, mice were housed individually, in white plastic cages (10 x 20 x 10 cm), received laboratory rodent chow (#5001) and water *ad libitum* and were maintained on a 16:8 h reversed L: D cycle with lights off at 0900. All procedures were performed

according to guidelines developed by the Canadian Council on Animal Care and protocols approved by the Dalhousie University Animal Care Committee.

Object Recognition Test. The object recognition test was performed as described by Ennaceur and Delacour (1988). The testing apparatus was an open field box (50 cm^3), with floors and sides of painted white plywood (without bedding material covering the floor). The mice were habituated to the testing apparatus for 5 min./day for 2 consecutive days. The object recognition test consisted of a familiarization phase followed by a test phase with a 15 min. inter-trial interval. During the familiarization phase, the mouse was allowed to explore two identical objects for 5 min and then returned to its home cage. Both objects were removed during the 15 min. interval and replaced with one identical and one novel object. The mouse was then placed back in the box for a 5 min. test phase. Both objects and object location were counterbalanced in order to remove any effect of object and/or location preference. The experimenter was blind to the identity of the subjects during the experiment and each trial was videotaped for later analysis. Exploration of an object was defined as directing the nose towards the object at a distance of 1 cm and/or touching the object with nose and paws. In order to obtain a measure of object discrimination, four measures were recorded; a) time (T) spent exploring object A and B during the familiarization phase (T1A, T1B) and b) time spent exploring the familiar object (A) and novel object (N) during the test phase (T2A, T2N). The discrimination ratio (T2N-T2A/(T2N+T2A)) was analysed by 2 (handling) x 2 (parental care) x 2 (gender) ANCOVA with initial exploration time (T1A + T1B) used as covariate.

Barnes Maze. The Barnes maze test of spatial learning and memory was based on that described by Pompl et al. (1999). We used a white circular platform (65 cm dia.) with 16 circular holes (4.45 cm dia.), 1.3 cm from the edge. Holes numbered 4, 8, 12, and 16 were escape routes with a white plastic escape box (29 x 13 x 13 cm) attached underneath the maze platform. Two 150-Watt lights and a buzzer were affixed 76 cm above the maze platform. The test procedure had 4 phases; habituation, acquisition, reversal, and a probe trial, and took 10 days to complete. On day 1, each mouse was randomly assigned to one of the four escape holes and habituated to the arena and to their appropriate escape hole by placing them over the escape hole, under a clear glass beaker, for 5 min. Acquisition took place on days 2-5 with reversal testing on days 6-9. Reversal testing consisted of moving the correct escape hole to the opposite quadrant of the apparatus (i.e. 4 becomes 8, and, 12 becomes 16). A 30 s probe trial was performed on day 10. Latency to find the escape hole, and the number of errors were recorded on each trial. An error was scored when the mouse's head dipped into an incorrect hole; repeated head dips to the same hole with no intervening behaviour were counted as one error. If, after 30 s, the mouse had not yet entered the escape box, a buzzer was turned on. Once the animal entered the escape box, the buzzer was turned off and the mouse was permitted to remain inside the box for 30 s. The maximum trial duration was 5 min. If the mouse did not enter the escape box after 5 min., it was guided to the correct hole. All trials were video taped for later analysis. Latency and number of errors made while searching for the escape hole were analysed by 2 (handling) x 2 (parental care) x 2 (gender) x 8 (day) repeated measures ANOVA. Percentage of time spent in the correct quadrant and number of approaches

to the correct escape hole during the probe trial were analysed by 2 (handling) x 2 (parental care) x 2 (gender) ANOVA.

Correlations. Based on the data acquired from the parental care observations of 28 litters of pups (n = 7 litters/treatment group), the mean percentage of observation periods with licking and grooming of pups was correlated with time spent exploring both objects during the familiarization phase, and the object discrimination ratio during the test phase of the object recognition test. The mean percentage of observation periods with licking and grooming of pups was also correlated with latency and number of errors made to find the escape hole during both acquisition and reversal training, percentage of time spent in the correct quadrant, and number of approaches to the correct escape hole during the probe trial in the Barnes maze. These measures were chosen because we wanted to assess the contribution of parental care toward different aspects of learning and memory. Correlations were performed using a simple linear regression analysis (Statview 5.0).

5.4. RESULTS- *Parental Care: Licking and Grooming.* There was a significant main effect of paternal presence ($F_{1, 21} = 9.96$, $p < .01$) on the percentage of observation periods with licking and grooming of pups during the postnatal period, as pups in the father-present group were licked and groomed more than those in the father-absent group. Posthoc analysis (Tukey/Kramer) revealed that on day block 4, there was no difference between handled father-present, handled father-absent, and nonhandled father-present groups in the percentage of observation periods with licking and

grooming of pups. There was a significant interaction between handling and day block on the percentage of observation periods with licking and grooming of pups ($F_{3, 63} = 7.16$, $p < .001$). Simple effects analysis revealed that handled pups received more licking and grooming over days ($F_{1,11} = 4.55$, $p < .05$), while the non-handled litters received less licking and grooming over days ($F_{1,11} = 5.49$, $p < .05$), (Figure 25a). Overall, there was a significant main effect of handling ($F_{1,21} = 6.40$, $p < .05$) on the average percentage of observation periods with licking and grooming of pups, as handled pups were licked and groomed more than non-handled pups (Figure 25b).There was a significant interaction between handling and paternal presence ($F_{1,21} = 10.09$, $p < .01$) on the average percentage of observation periods with licking and grooming of pups. Handled, father-present pups received the most LG while non-handled, father-absent pups received the least LG.

Figure 1

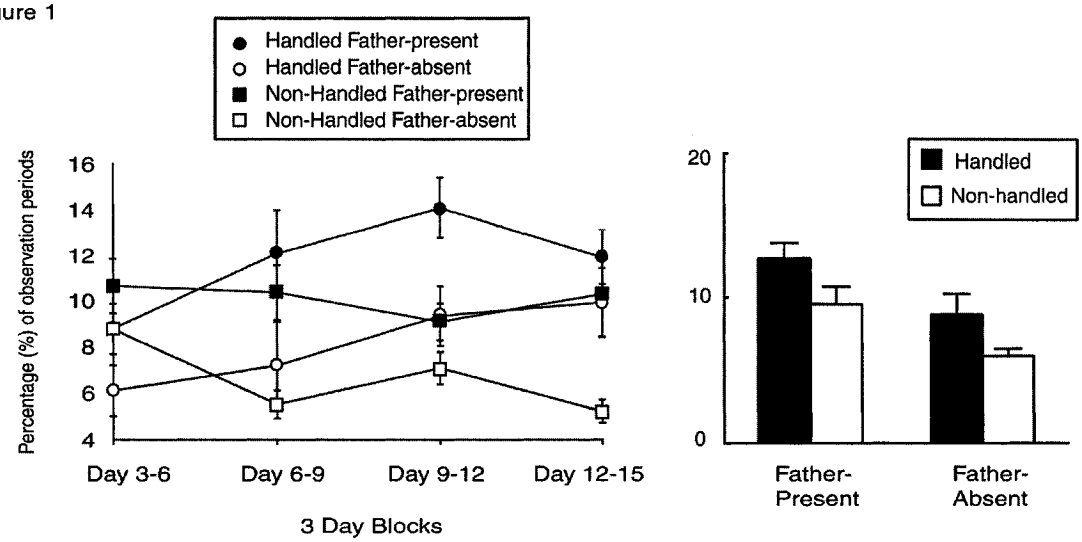


Figure 25. (a) Mean ± SEM percentage of observation periods in which licking and grooming (LG) were received by offspring from postnatal days 3-15 in handled, and non-handled, biparental, and father-absent families (n=6-7/group).

Object Recognition Test. There was a significant main effect of gender ($F_{1, 70} = 4.84$, $p < .05$) on total exploration in the object recognition test as females spent more time exploring all objects than males (Figure 26a). There was a significant main effect of phase ($F_{1, 70} = 4.28$, $p < .05$) on exploration as animals explored both objects more during the choice phase of the object recognition test (Figure 26a). In order to account for any group variability in baseline exploratory activity, we used the total time spent in contact with both objects during the familiarization phase (T1A+T1B) as a covariate for analysis of the novel object discrimination ratio in the choice phase. ANCOVA revealed a 3-way interaction between handling, paternal presence and gender ($F_{1, 62} = 5.38$, $p < .05$) on novel object discrimination. Relative to chance, handled, father-present males showed and increase in object discrimination. There was no effect in females (confidence interval: $t_{\alpha .05} = 2.26$, $0.78 \geq \mu \geq 0.54$). There was no effect of paternal presence or gender on object recognition in non-handled animals (Figure 26b).

Figure 2

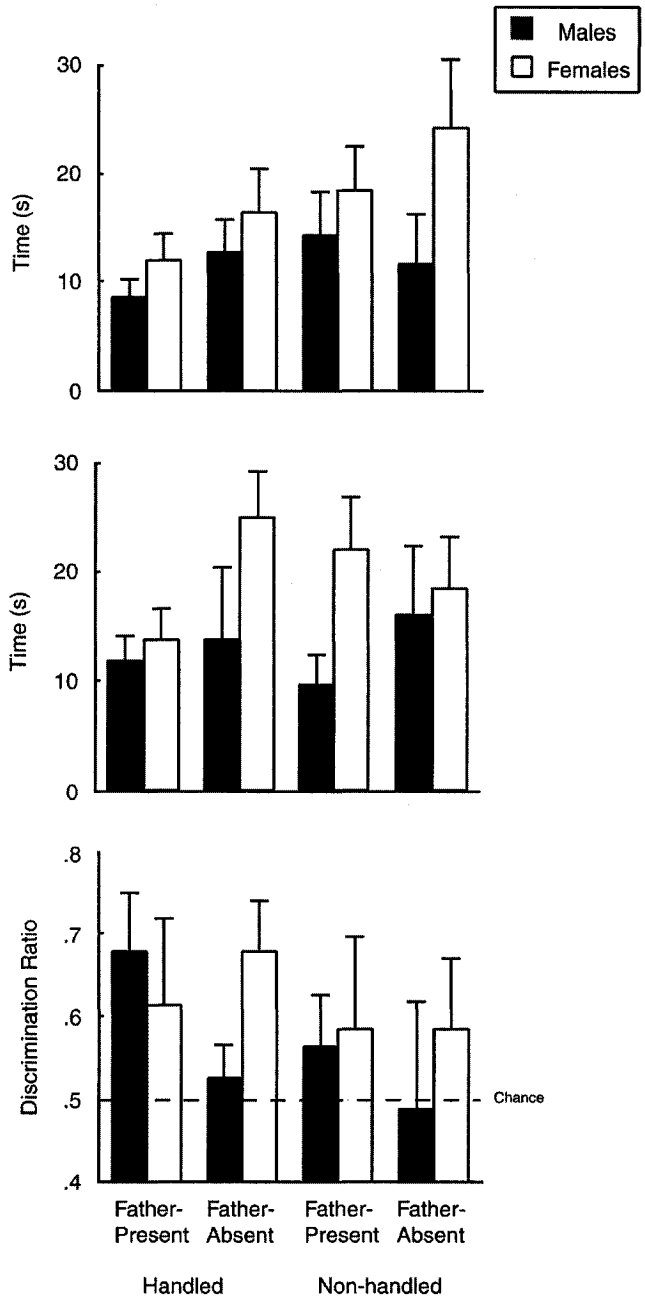


Figure 26. Mean ± SEM time (s) spent exploring both objects during (a) the familiarization and (b) choice phases of the object recognition test (n=9-10/group).

Barnes Maze. Overall, there was a significant main effect of handling ($F_{1, 70} = 14.05$, $p < .001$) on latency to find the escape hole in the Barnes maze as handled mice had shorter latencies to find the escape hole over all days of testing than non-handled mice (Figure 27a). There were no significant main effects of paternal presence or gender on latency to find the escape hole in non-handled animals. As expected, latency to find the escape hole decreased for all groups over days ($F_{7, 490} = 21.52$, $p < .0001$). Due to the strength of the handling effect and the way ANOVA partitions group variance, the overall ANOVA was unable to detect any interaction. However, after observing the histogram for latency to find the escape hole, it was obvious that within the nonhandled group, father-absent females performed like handled mice. For this reason, we went ahead and performed basic post-hoc analyses using the very conservative Sheffes test. Post hoc analysis revealed that, in the non-handled group, father-absent females were significantly different from the other three non-handled groups (all p's $< .05$) and not significantly different from any of the four handled groups (Figure 27a).

There was a significant main effect of handling on the number of errors in the Barnes maze ($F_{1,64} = 6.21$, $p < .05$) as handled mice made fewer errors than non-handled mice while finding the escape hole over all days of testing (6 animals were excluded from this repeated measures analysis because they froze or jumped off the apparatus during testing) (Figure 27b). There was no significant effect of paternal presence or gender on number of errors in non-handled animals. As expected, the number of errors decreased for all groups over days ($F_{7, 448} = 12.64$, $p < .0001$). On day 5, the first day of reversal training (and the most relevant for cognitive

performance), there was a significant main effect of handling on the number of errors ($F_{1,66} = 5.68$, $p < .05$) as handled mice made fewer errors than non-handled mice while finding the escape hole (4 animals were excluded from this 3 way analysis because they froze or jumped off the apparatus during testing (see Figure 27b). There was no significant effect of paternal presence or gender on number of errors on day 5 in non-handled animals.

Figure 3

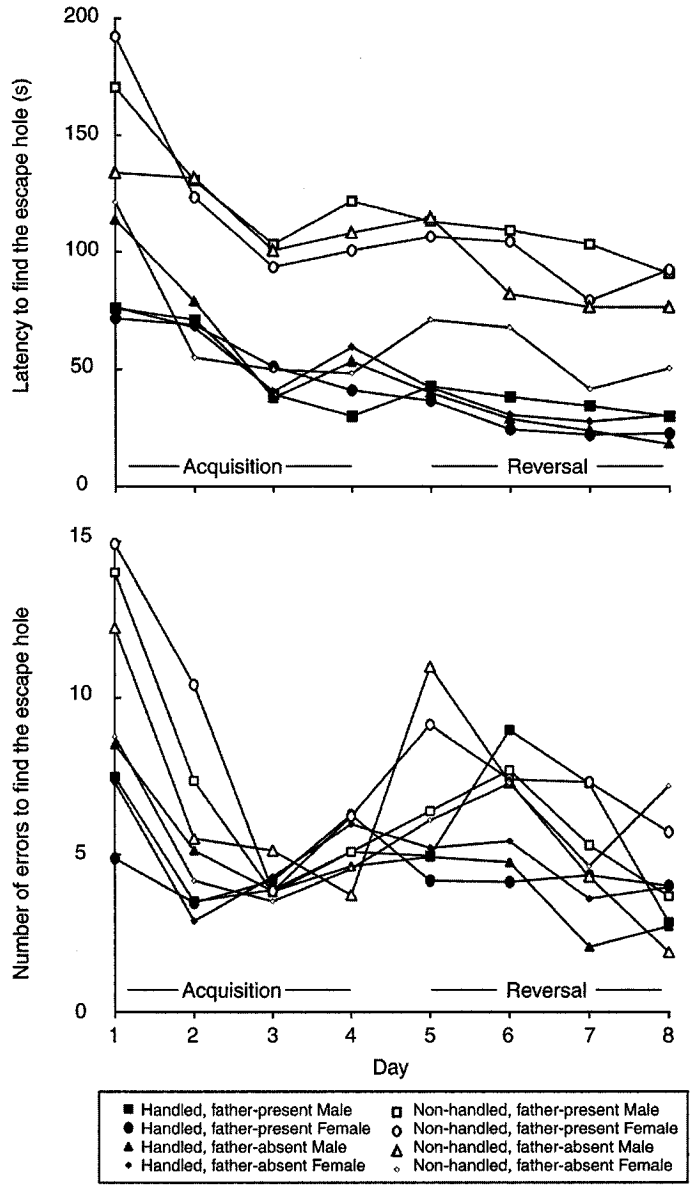


Figure 27. a) Mean ±SEM latency (s) to find the escape hole across 8 days during the Barnes maze test of spatial learning. b) Mean ±SEM number of errors to find the escape hole across 8 days during the Barnes maze test of spatial learning.

There were no significant main effects of handling, paternal presence, or gender on the percentage of time spent in the correct quadrant of the Barnes maze during the probe trial (Figure 28a). There was a marginally significant main effect of handling on number of approaches ($F_{1,70} = 3.33$, $p = .07$) as handled mice made more approaches to the correct escape hole than non-handled mice during the probe trial (Figure 28b). Simple effects analysis revealed that, in male mice, there was a significant main effect of handling on number of approaches ($F_{1,35} = 7.16$, $p < .05$) as handled male mice made more approaches to the correct escape hole during the probe trial than nonhandled males (Figure 4b). In non-handled, male mice, paternal presence had no significant effect on probe trial performance. In female mice, there was no significant effect of handling or paternal presence on the number of approaches to the correct escape hole during the probe trial.

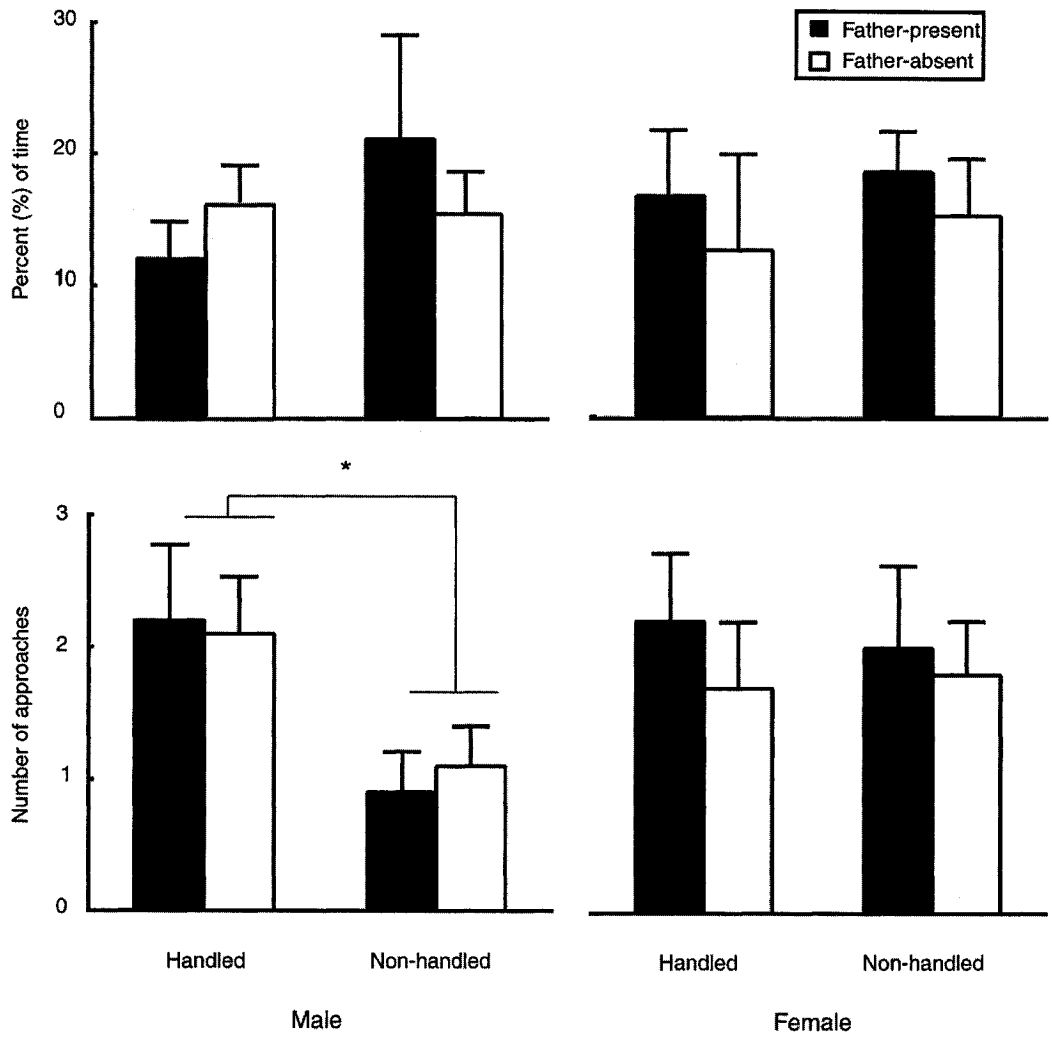


Figure 28. (a) Mean ± SEM percentage of time spent in the correct quadrant during the probe trial. There were no significant differences. b) Mean ± SEM number of approaches made to the correct escape hole in the probe trial. Handling increased approaches to the correct escape hole in male mice with no effect in females ($p<.05$) (Bredy et al., in press).

Parental care and offspring performance correlations. There was no significant correlation between the mean percentage of observation periods with licking and grooming of pups and exploration time in the familiarization phase, or discrimination ratio during the choice phase of the object recognition test. In the Barnes maze, there was no significant correlation between the mean percentage of observation periods with licking and grooming of pups and latency or errors made to find the escape hole during acquisition, or reversal training in the Barnes maze. However, there was a significant negative correlation ($r = -33$, $F_{1,,58} = 6.80$, $p < .05$) between licking and grooming and the number of errors made on the first day of reversal training (19 mice were not analysed because we did not have parental care data for them) (Figure 29). In males ($n = 29$), there was no significant correlation between licking and grooming and number of errors, while in females ($n = 30$), the correlation was significant ($r = -.47$, $F_{1, 29} = 7.94$, $p <. 01$). There was no significant correlation between licking and grooming and percentage of time spent in the correct quadrant during the probe trial and no significant correlation with the number of approaches to the correct escape hole during the probe trial.

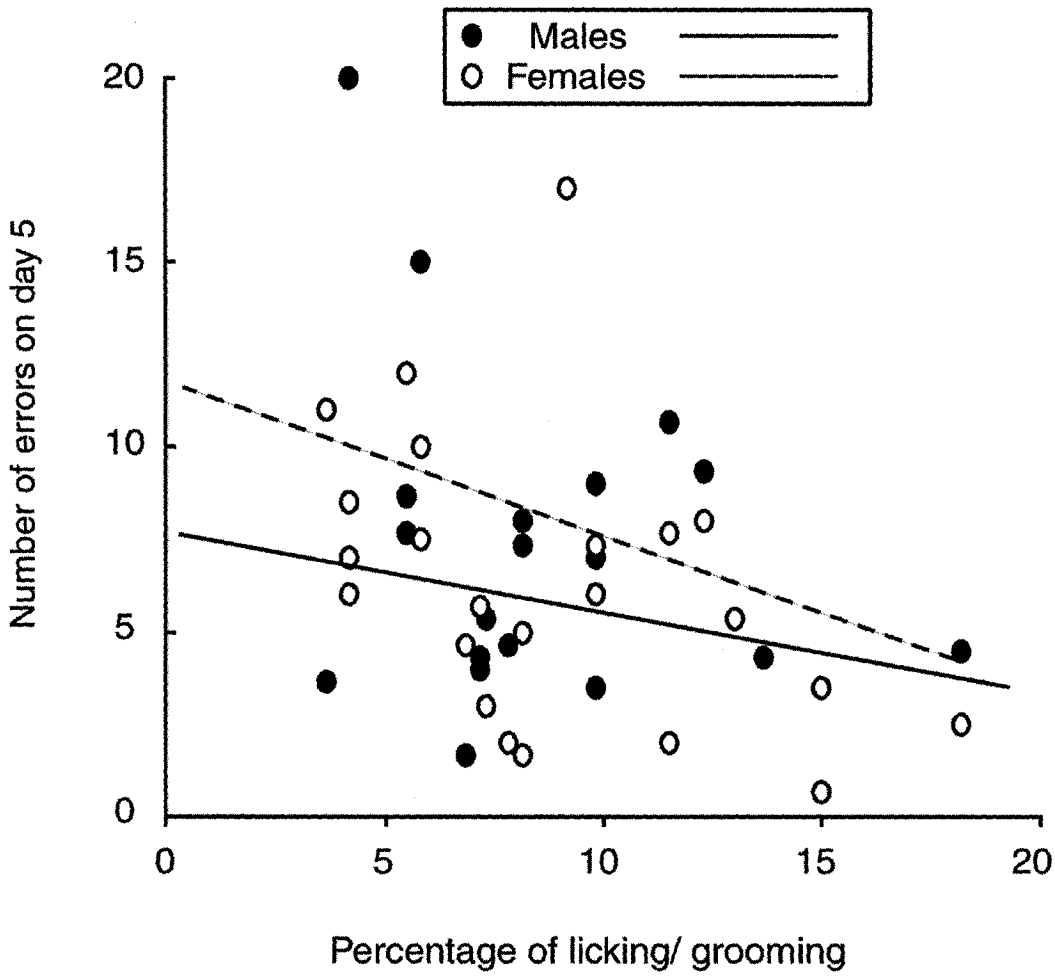


Figure 29. Scatterplot of the relationship between percentage of observation periods of licking and grooming received by offspring and number of errors made while searching for the escape hole on the first day of reversal training (n = 59).

5.5. DISCUSSION- The current study was designed to examine the interactions between neonatal handling and biparental behaviour during the early postnatal period and their effects on cognitive function in adult *P. californicus*. There were 3 notable findings: 1) Paternal presence and neonatal handling influenced the amount of licking and grooming received by pups from their parents during the first 15 postnatal days; 2) Neonatal handling of *P. californicus* pups enhanced spatial learning and memory in adulthood; and, 3) There were gender differences in the effects of the interaction between neonatal handling, and paternal presence on adult learning and memory. The effects appeared to be associated with the presence of the father under specific environmental conditions.

Neonatal handling effects parental care. Male and female *P. californicus* spend approximately equal time performing all components of parental behaviour, with the exception of maternal arched-back nursing and lactation (Dudley, 1974b; Gubernick and Alberts, 1987). As expected, handled and non-handled pups reared with both parents received more licking and grooming than those reared with the mother alone. Neonatal handling increased licking and grooming such that by postnatal day 12-15, licking and grooming of handled, father-absent pups was equivalent to that of pups in non-handled, father-present families (Figure 25). These data are consistent with previous studies that show increased maternal care in the rat as a function of handling (Lee and Williams, 1975; Villescas et al., 1977; Liu et al., 1997; D'Amato et al., 1998; Pryce, Bettschen and Feldon. 2001). Not only do these data support the hypothesis of Levine (1967) that increased parent-infant interaction occurs in

response to handling, but they also extend the earlier findings to include the monogamous, biparental, California mouse.

Neonatal handling effects offspring cognitive performance. As predicted, neonatal handling enhanced spatial learning in adulthood by reducing the latency, and number of errors made, in finding the escape hole in the Barnes maze (Figure 27). On day 1 of acquisition, there was a clear difference between handled and non-handled mice in their latency to find the escape hole. An examination of the strategies (random, direct, or serial searching pattern) used to find the escape hole revealed no group differences (data not shown) suggesting that there were no differences in motivation to find the escape hole. Furthermore, since the mice were habituated to the task prior to testing, the differences in performance on the first day cannot be explained on the basis that they differ in reactivity to novelty. Thus, the data support a previous study demonstrating that exposure to novelty (such as that associated with handling) during the neonatal period enhances spatial learning and memory in the Morris water maze (Tang. 2001). Further, if we consider neonatal handling to be a form of early environmental enrichment, the data also supports studies where animals with enriched experience show enhanced performance in spatial learning tasks (Falkenberg et al., 1992; Kemperman et al., 1997; Rampon et al., 2000; Bredy et al., 2003).

Contrary to our hypothesis, increased licking and grooming provided by the father in the non-handled, father-present, group did not enhance spatial learning. These data suggest that, under standard laboratory conditions, the presence of the father may not enhance offspring cognitive development. In the rat, it appears that the

critical feature of maternal care isn't such that rat pups who receive more licking and grooming necessarily exhibit enhanced cognitive development, rather, deficits in development are more strongly related to reductions in maternal licking and grooming (M.J. Meaney, personal communication). In essence, we may be observing a "ceiling effect" with respect to the positive influence of parental licking and grooming on cognitive development. This issue remains to be clarified. Furthermore, Cantoni and Brown (1997) observed no effect of paternal presence on offspring survival and development when mothers were allowed to rear their offspring with food and water provided *ad libitum*. In fact, paternal presence had more effects on the mother than on the pup's development. However, if parents are required to work for food, the presence of the father does enhance pup survival and development (Cantoni and Brown, 1997; Wright and Brown, 2002). Taken together, the findings suggest that paternal care may facilitate offspring development under conditions of increased environmental demand when the mother may neglect pups. Under standard lab conditions pup development may be maximized (a ceiling effect) and male parental care can, therefore, have no significant effect (Brown, 1993).

Gender specific responses to the effects of neonatal handling and paternal care. In contrast to our null hypothesis, neonatal handling and paternal presence had variable, gender-specific effects on offspring spatial and non-spatial learning in adulthood. Handling enhanced object recognition in father-present but not in father-absent males, with no effect in females (Figure 26b). Paternal presence effects emotional development in male mice and, in rats, performance in the object recognition test is

dependent on an animal's emotional response to novelty (Mugford and Nowell, 1973; Renner, Bennett and White, 1992; Tomihara, 2001). The data suggest that in *P. californicus*, where both parents invest in the development of the offspring, the presence of the father may be important for mediating the effects of handling on reaction to changes in the environment in male offspring. An obvious issue that remains to be addressed is why female mice did not show enhanced object recognition learning in response to neonatal handling.

In the Barnes maze, there was a negative correlation (r = -.33) between parental licking and grooming and number of errors made before finding the escape hole on the first day of reversal training (Figure 29) and this correlation was strongest in female mice (r = -47). The most interesting finding to emerge from the spatial learning data was that non-handled, father-absent, females performed as well as mice that had been handled during the postnatal period (Figure 27). Under stable laboratory conditions, mother rats normally show a bias towards caring for male offspring (Moore and Morelli, 1979). However, parental investment theory suggests that maternal care strategies are quite sensitive to changes in the environment (Wells, 2000). For example, in humans, environmental stress, such as low socio-economic status, results in increased maternal investment favouring female offspring (Koziel and Ulijaszek, 2001). Moreover, social stress, such as that imposed by low social rank, results in a sex-bias of maternal investment towards female offspring in rhesus macaques (Schino, Cozzolino and Troisi, 1999; Maestripieri, 2001). Studies in human children have shown that the quality of maternal care received early in life contributes to language and cognitive development (Ruddy and Bornstein, 1982; Bornstein,

1985). Perhaps, as a result of the stress of handling and/or the absence of the father in the neonatal environment, the mother-infant interaction changed such that the course of cognitive development was altered by a bias in maternal care towards her female offspring. This line of reasoning warrants further investigation.

An issue that needs be clarified is the relationship between parental licking and grooming and spatial and non-spatial learning in male mice from intact families. When performing parental observations on intact families, we did not differentiate between maternal- and paternal-licking and grooming nor did we determine the amount of licking and grooming received by male or female pups. Future studies will determine if there is a gender bias in maternal- and paternal- licking and grooming of the offspring in response to neonatal handling and/or the absence of paternal investment in *P. californicus*.

5.6. CONCLUDING REMARKS- The current study supports previous findings that variations in parental care occur as a result of neonatal handling and that changes in parental care can contribute to offspring cognitive performance in *P. californicus*. The data suggest that environmental determinants modulate, in a gender specific manner, the effects of biparental care on offspring cognitive development. The importance of paternal investment may only become clear when environmental demands are such that maternal care is compromised. Finally, the data provide a basis for future studies on the role of paternal investment and biparental care for offspring development.

CHAPTER 6- GENERAL DISCUSSION- ENVIRONMENTAL ENRICHMENT REVERSES THE EFFECT OF REDUCED MATERNAL CARE IN EARLY LIFE: IS IT REVERSAL, OR SIMPLY COMPENSATION?

6.1. HISTORICAL OVERVIEW- Since the end of the 18th century, neuroscientists have questioned whether experience can enhance brain and behavioural development. When M.V. Malacarne (1744-1816) investigated the effect of environmental enrichment on the central nervous system in birds, he noted that those that had received enriched training had larger brains than their non-enriched counterparts from the same clutch (cited in Renner and Rosenzweig, 1987). Systematic experimental research on the influence of environmental enrichment upon learning and memory truly began 50 years ago when D.O. Hebb, while at McGill University, described how enrichment throughout the peripubertal period enhanced maze learning in the rat (Hebb, 1949).

It is now commonly accepted that the environmental conditions in which animals are housed produces neuroanatomical changes and significantly affects behaviour (for reviews, see Renner and Rosenzweig, 1987; Rampon and Tsien, 2000). Experience has a powerful influence on brain anatomy, such that environmental enrichment increases total brain weight and cortical thickness, increases cortical synapse-to-neuron ratio, and increases dendritic branching (Diamond, 1988; Beaulieu and Colonnier, 1987; Greenough and Volkmar, 1973). Animals exposed to enrichment also show facilitated hippocampal long-term potentiation, increased expression of neurotrophic factors BDNF and NGF, and protection from neuronal death in the brain (Falkenberg et al., 1992; Pham et al.,

1999; Young et al., 1999; van Praag et al., 2000; Duffy et al., 2001). Environmental enrichment enhances learning and memory in various behavioural tasks, including spatial memory in the Morris water maze and recognition memory in the novel object recognition task (Falkenberg et al., 1992; Kempermann et al., 1997; Kempermann and Gage, 1999; Rampon et al., 2000b). Environmental enrichment consists of several components, including increased social interaction, greater physical activity, and expanded learning opportunities. The effect of environmental enrichment is not mediated by increased motor activity alone, for example, social interaction and environmental complexity appear to be most critical in determining increases in dendritic remodelling in the hippocampal formation and subsequent changes in learning (Berman et al., 1996; Faherty et al., 2003).

In the late 1950's, Cooper and Zubek introduced the possibility that environmentally-induced and/or inherited behavioural traits could be reversed with enrichment, and that there existed individual differences in sensitivity to environmental experience. Among animals selectively bred for performance in the Hebb-Williams maze, enrichment improved performance of 'maze-dull' rats, with no appreciable effect on 'maze bright' rats (Cooper and Zubek, 1958). In another study, they demonstrated that glutamic acid supplementation during early development improved cognitive performance, an effect that was also specific for 'maze-dull' rats (Hughes and Zubek, 1956). Lewis Bernstein further promoted the idea of reversibility by showing that environmental experience could reverse T-maze performance deficits in isolated rats (Bernstein et al., 1960). This compensation could be induced by later enrichment and was extended to include learning and memory in the Lashley III

maze, suggesting that enrichment reverses the effect of early isolation on generalized learning ability (Bernstein, 1971; Bernstein, 1972). In the latter of these studies, the concept of timing and duration of environmental enrichment was introduced. Exposure to a free exploratory environment could produce the same effects as practice, and, if the duration of enrichment was a long enough, could overcome the effects of early isolation (Bernstein, 1972). The studies by Hebb, Bernstein, Cooper and Zubek could be considered the first to demonstrate the importance of adolescence as a critical period in which brain development is particularly sensitive to the effects of environmental stimuli.

The subject of reversibility and individual sensitivity resurfaced during the 1990s with the use of the Swiss line of Roman high- and low-avoidance (RHA/Verh and RLA/Verh) rats, selectively bred for rapid (RHA/Verh) versus poor (RLA/Verh) acquisition of avoidance learning. Like the McGill 'maze bright' and 'maze dull' lines, these rats showed a selective sensitivity for the beneficial effects of environmental enrichment on learning and memory; the RLA/Verh line exhibits a marked responsivity to enrichment, with little or no effect in the RHA/Verh line (Fernandez-Teruel et al., 1997; Fernandez-Teruel et al., 2002). These pioneering studies on the selective effects of environmental enrichment and individual differences in sensitivity to enrichment provided the basis for the fundamental questions addressed and presented in this thesis.

6.2. ENRICHMENT REVERSES THE EFFECT OF REDUCED MATERNAL-LG ON COGNITIVE FUNCTION- Exposure to environmental enrichment during

adolescence reversed hippocampal-dependent learning deficits in the offspring of Low-LG mothers (Figures 14 and 15). The effect of prenatal stress, prenatal alcohol exposure, and postnatal lead exposure on spatial learning can all be reversed through environmental enrichment during the adolescent period (Lee and Rabe, 1999; O'Leary et al., 2002; Koo et al. 2003, Guilarte et al., 2003).

The beneficial effects of enrichment on hippocampal development and function are not limited to the peripubertal period, the effect of social isolation from postnatal days 22-49 on spatial learning, hippocampal neurogenesis, and synaptic plasticity in area CA1 can be reversed by subsequent enriched social housing conditions from postnatal 50-77 (Lu et al., 2003). Further, while exposure to enrichment during adolescence has the strongest effect on cognitive function, both 11 and 22 month old rats housed in enrichment also show enhanced cognitive function (Kobayashi et al., 2002). Taken together, these studies provide substantial support for the idea that the effects of early life adversity on cognitive development need not be permanent, and support the notion that the hippocampal formation retains its characteristic plasticity well into adulthood. The critical question concerns the mechanism of these enrichment-induced changes in cognitive function.

6.3. NEURAL MECHANISMS OF ENRICHMENT-INDUCED REVERSAL OF COGNITVE DEFICITS ASSOCIATED WITH REDUCED-LG- Environmental enrichment during adolescence reversed the effect of reduced maternal care on genomic targets known to contribute towards hippocampal-dependent learning and synaptic plasticity. Enrichment reversed the effect of reduced maternal care on the

mRNA expression of the NR2A and NR2B subunits of the NMDA receptor complex, with the pronounced effects occurring in area CA1 of the hippocampus (Figures 20 and 21). Enrichment also reversed the effect of Low-LG on GluR1 and GluR3 mRNA expression in the right hippocampal area CA1 (Figures 22 and 23). As with the behavioural findings, the influence of enrichment was specific to the offspring of Low-LG mothers, with no clear effect on the offspring of High-LG mothers.

The NMDA receptor complex, and the NR2B subunit in particular, is interesting because of its established role in facilitating synaptic plasticity and enhancing hippocampal-dependent learning (Tang et al., 1999). The effect of environmental enrichment on spatial learning can be blocked with the NR2B subunit specific antagonist Ifenprodil (Lee et al., 2003). Further, anti-sense knockdown of hippocampal NR2B mRNA expression with an accompanying decrease in protein expression can mimic age-induced impairments in synaptic plasticity and spatial learning (Clayton et al., 2002). Taken together with the finding that a single infusion of Ifenprodil eliminates the effect of increased maternal care on spatial learning (Figure 24), these data strengthen the argument for the importance of the NMDA receptor complex, and particularly the NR2B subunit, in mediating both the effect of maternal care and environmental enrichment on hippocampal development and function. Interestingly, exposure to dexamethasone (a synthetic glucocorticoid) across the neonatal period leads to a decline in hippocampal NR2B subunit protein levels in adulthood, an effect accompanied by impairments in spatial learning and memory (Kamphius et al., 2003). This finding becomes particularly important in the next

section when we consider the influence of maternal care and enrichment on stress reactivity and its contribution to learning and memory processes.

One of the first studies to elucidate the molecular targets mediating the effect of enrichment was performed using a gain of function approach. Under standard housing conditions, transgenic mice over-expressing the NR2B subunit in the forebrain exhibit larger hippocampal LTP and superior learning and memory compared to wild-type controls (Tang et al., 1999). Tang et al. (2001) used these NR2B transgenic mice to address gene by environment interactions associated with hippocampal-dependent learning. After exposure to environmental enrichment, wild-type mice showed an overall improvement in contextual and cued conditioning, fear extinction and novel object recognition learning. While enrichment was unable to further increase the performance of NR2B transgenic mice in contextual and cued conditioning, or fear extinction, there were additive effects of gene manipulation and enrichment on novel object recognition. Forebrain protein expression for the NR2A, NR2B, and GluR1 subunits were increased in both wild type controls and NR2B transgenic mice. The data suggest that environmental enrichment can enhance learning and memory through effects on multiple targets in the forebrain, through specific subunits of the hippocampal glutamate receptor, with particular emphasis on the role of the NR2B subunit of the NMDAR complex.

Lee et al. (2003) found that environmental enrichment-induced increases in SGK (a serum and glucocorticoid-inducible kinase implicated in memory consolidation of spatial learning, Tsai et al., 2002) are mediated by the activity of AMPA receptors in area CA1 of the hippocampal formation. The immediate

impression after examining these data was that there appear to be two separate pathways involved in the effect of environmental enrichment on hippocampal-dependent learning and function. First, recall the NMDAR pathway, changes of which are involved in facilitating synaptic communication during the acquisition of novel information. In some tasks, prior exposure to elements of the testing environment attenuates the effects of NMDAR antagonists. Thus, the effects of NMDAR blockade on hippocampal-dependent learning may ultimately depend on familiarity with environment. For example, although NMDA antagonists impair learning in the radial arm maze, Morris water maze, and contextual fear conditioning tasks (Kawabe et al., 1998; Alhander et al., 1999; Sanders and Fanselow, 2003), adult rats can perform successfully provided they are exposed to the strategies required to solve the task prior to drug administration (Shapiro and O'Connor, 1992; Saucier and Cain, 1995; Sanders and Fanselow, 2003). Conversely, NMDA antagonists block the enhancing effects of novelty on retrieval of a previously learned task (Izquierdo et al., 2001).

Given the evidence that NMDARs serve as coincidence detectors, primarily involved in reaction and learning in response to novelty (Wittenberg and Tsien, 2002, Quinlan et al., 2004), these studies emphasize the importance of stable NMDAR activity during initial responses to environmental cues, hence a role for the hippocampal NMDAR in processes of adaptation, and their importance for hippocampal-dependent learning and memory. Second, AMPARs appear to be involved in the consolidation of spatial memory, through their interaction with SGK and the glucocorticoid pathway, thus an intimate relationship between AMPAR and

the stress may also contribute to hippocampal-dependent learning and memory (see Roozendaal, 2002; Tsai et al., 2002; Lee et al., 2003).

6.4. ALTERNATIVE HYPOTHESIS: STRESS, GLUCOCORTICOIDS, UAND HIPPOCAMPAL-DEPENDENT LEARNING- Considering the role of the AMPAR and its interaction with SGK and the glucocorticoid pathway, one can surmise that the effect of environmental enrichment during adolescence and its ability to reverse cognitive deficits associated with reduced maternal care may be mediated, in part, by changes in neural systems associated with fearfulness and anxiety.

Under standard housing conditions, the offspring of Low-LG mothers are more fearful, have a greater pituitary-adrenal response to stress, and perform poorly in the Morris water maze by comparison with the offspring of High-LG mothers (Caldji et al., 1998; Liu et al., 1997; Liu et al., 2000). Not surprisingly, the offspring of Low-LG mothers exhibited increased thigmotaxis in the Morris water maze, a common fear response in rodents to novel environments (Holscher et al., 1999). Performance differences in the water maze are associated with thigmotaxis, the reduction of which is fundamental for successful acquisition of the water maze task (Hoh et al., 1999; Wishaw, 1989). Predictably, the offspring of Low-LG mothers reared under enriched conditions showed decreased thigmotaxis and improved performance in the water maze task (Figure 30).

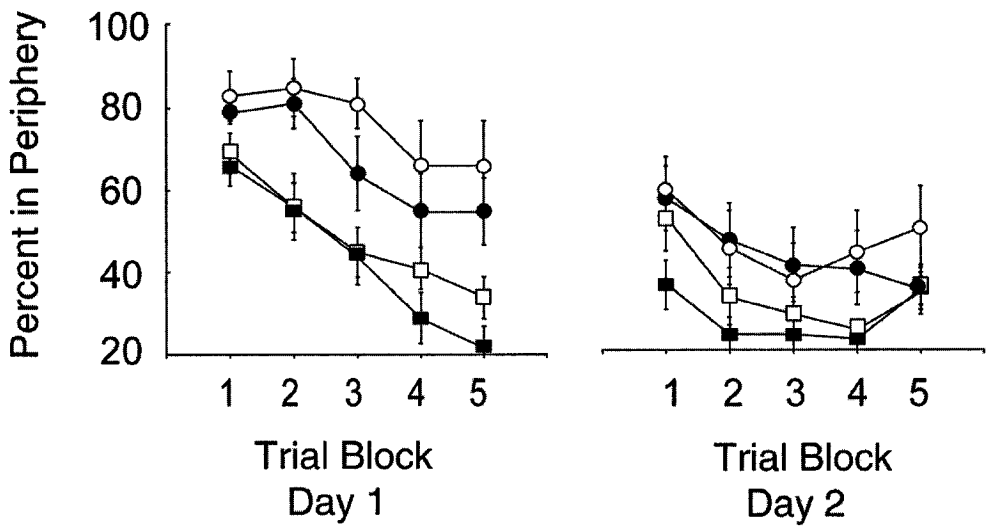


Figure 30. There was a significant main effect of housing ($F_{1,64} = 34.83$, $p < .0001$) and day ($F_{1,64} = 36.98$, $p < .0001$) on percent of time spent in the outer region of the swim pool. Enriched rats showed less thigmotaxis than standard-housed rats and thigmotaxis decreased for all groups across days. Post-hoc analysis (Tukey/Kraemer test) showed that enriched rats spent less time in the periphery on all trial blocks (all p's $< .01$). There was a significant maternal care by housing interaction ($F_{1,64} = 4.63$, $p < .05$), with the standard-housed offspring of High-LG mothers spending significantly less time in the periphery than the offspring of Low-LG mothers, a difference completely eliminated under conditions of enrichment.

Environmental enrichment increases hippocampal GR mRNA expression, lowers basal level and mild stress-induced increases in ACTH and corticosterone release, and reverses the effect of early postnatal maternal deprivation on fear-related behaviour and pituitary-adrenal responses to stress (Olsson et al., 1994; Francis et al., 2002; Belz et al., 2003). The offspring of Low-LG mothers show decreased hippocampal GR mRNA expression compared to the offspring of High-LG mothers (Liu et al., 1997), a difference that is eliminated in the right hippocampus when the animals are reared under conditions of environmental enrichment (Figure 31).

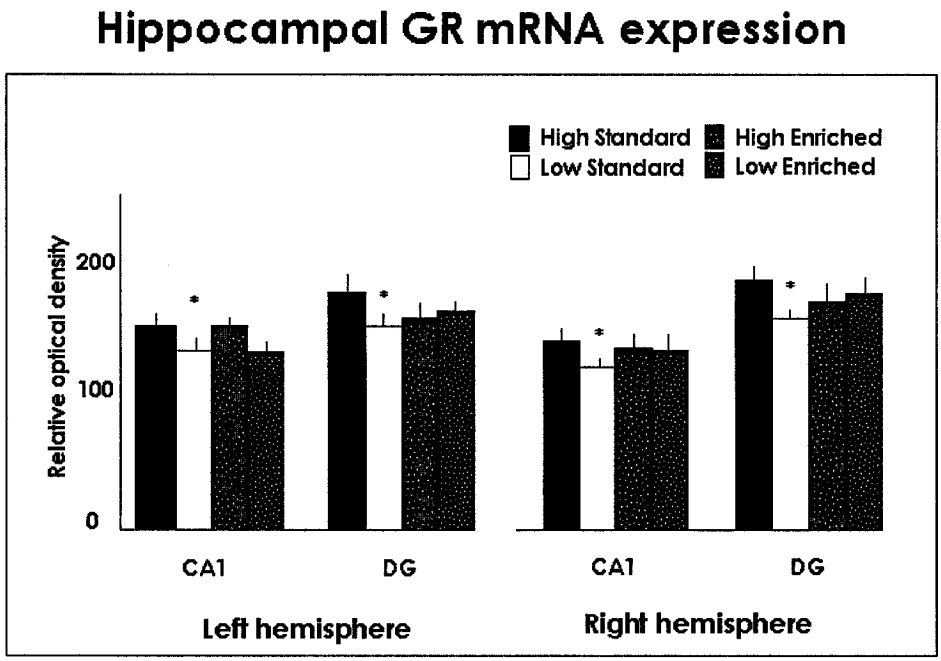


Figure 31. There was a significant main effect of region ($F_{1,72} = 14.65$, $p < .001$) and maternal care ($F_{1,72} = 7.86$, $p < .01$) on GR mRNA expression in the hippocampus. Overall, there was more GR mRNA expressed in the hippocampal formation in the DG and offspring of High-LG mothers showed more GR mRNA expression throughout the hippocampal formation. There was a significant maternal care by housing interaction ($F_{1,72} = 4.24$, $p < .05$), as enrichment increased GR mRNA expression in the offspring of Low-LG mothers in right hippocampus while decreasing GR mRNA expression in the DG of High-LG offspring (n = 5-6/group).

The effect of enrichment on GR mRNA expression in the Low-LG offspring is very interesting considering it is lateralized, as with the AMPA mRNA expression, to the right hippocampus. Denenberg et al., (1978) demonstrated that early life experience leads to cerebral lateralization and functional asymmetry in the rat. Rats that had been 'handled' during the early postnatal period were more exploratory in the open field test, effects that were eliminated with lesions of the right cerebral cortex. Further, these data were extended to include the hippocampal formation by the finding that exposure to novelty during the neonatal period leads to both a volumetric asymmetry and a selective enhancement of synaptic plasticity in the right hippocampus, effects accompanied by enhanced spatial learning and memory (Verstynen et al., 2001, Tang and Zou, 2002; Tang, 2001). To date, the data showing a right hippocampus specific effect of environmental enrichment on AMPAR subunit and GR mRNA expression in the offspring of Low-LG mothers is the first to demonstrate asymmetric brain development in response to environmental stimulation during adolescence.

It has been shown that enrichment reduces fear responses to novelty, and the effects are especially notable in more anxious animals (Chapillon et al., 1999; Fernandez-Teruel et al., 1997; Francis et al., 2002). Continuing with the theme of reactivity to novelty, cognitive ability in aged rats can be predicted by their reactivity to novelty early in life (Dellu et al., 1994). High reactive (HR) rats show a higher corticosterone response to stress than low reactive LR rats and very interestingly, young HR rats show cognitive impairment after exposure to chronic social stress, with no effect on LR rats (Dellu et al., 1996; Touyarot et al., 2004). Renner et al.,

(1992) showed that object recognition is impaired by fear of novelty and enrichment completely eliminated the effect of maternal care on performance in this task (Figure 13).

Taken together with our observations on object recognition and thigmotaxis, environmental enrichment may reverse cognitive deficits associated with reduced maternal care, in concert with the bilateral effects on the NMDAR-coincidence detector, through compensatory effects on fearfulness and anxiety that involves an AMPA-GR interaction in area CA1 of the right hippocampus. Both pathways would contribute to the interaction between emotionality and cognitive function that is indistinguishable when it comes to their participation in hippocampal-dependent learning and memory.

6.5. STRUCTURAL REMODELLING MAY CONTRIBUTE TO THE EFFECT OF ENRICHMENT- Dendritic remodelling is involved in the establishment of circuitry of the hippocampus during development, and in activity- and experience-dependent neural plasticity. Environmental enrichment increases hippocampal synaptophysin expression and increases synaptic efficacy in an *in vitro* model of LTP (Foster et al., 2000; Saito et al., 1994). Further, rats exposed to enrichment also show increased synaptic vesicle density and enhanced hippocampal synaptic plasticity (Mollgaard et al., 1971; Nakamura et al., 1999). The offspring of Low-LG mothers show decreased hippocampal synaptophysin expression by comparison with the offspring of High-LG mothers, a difference that is eliminated after exposure to environmental enrichment (Figure 19). These findings are similar to those of Koo et

al. (2003) who found that enrichment reversed the effect of prenatal stress on hippocampal synaptophysin expression. The effect of chronic social stress in the HR rats is also associated with decreased hippocampal synaptogenesis (Touyarot et al 2004), thus, in conjunction with our data on synaptophysin expression, the evidence is strong in favour of the idea that changes in structural remodelling in the hippocampus contributes to differences in behavioural reactivity to novelty and subsequent cognitive ability.

The hippocampal formation is one of the most unique regions of the brain because of its ability to continually regenerate new neurons across the lifespan. Stress hormone levels and behavioural inhibition in response to novelty are negatively correlated with hippocampal cell proliferation and survival, as well as spatial learning (Gould et al., 1999; Lemaire et al., 1999; Lemaire et al., 2000; Ennaceur et al., 1997). Glucocorticoids regulate survival of adult generated neurons such that low levels of corticosterone enhance while high levels of corticosterone diminish neuron survival in the adult hippocampus (Wong, Herbert and Harris, 2003; Karishma,and Herbert, 2003). Thus, given what we know about stress reactivity as a function of maternal care and environmental enrichment, it is not surprising that the offspring of High-LG mothers have enhanced survival of newborn neurons across the lifespan (Figure 7). Whether or not neuronal survival in adulthood can be enhanced in the Low-LG offspring after exposure to peripubertal enrichment, and whether such effects would be functionally relevant, remains to be determined. Environmental enrichment reverses the effect of prenatal stress on spatial learning and this effect is associated with increased neuronal proliferation and survival, and enhanced synaptogenesis

(synaptophysin and N-CAM expression) (Koo et al., 2003). Based on these findings, it seems plausible that environmental enrichment during adolescence will rescue cognitive deficits associated with reduced maternal care through effects on structural (both synaptic and neuronal) remodelling in the hippocampal formation.

6.6. NEUROTROPHIC FACTORS- WHAT WE KNOW AND WHERE WE'LL GO- Neurotrophic factors, originally identified as target-derived signals that regulate survival and differentiation of neurons, are now known to support neuronal excitability, synaptic development, and maintenance of synaptic plasticity (reviewed in Schinder and Poo 2000; Munno and Syed, 2003). Under standard housing conditions, the offspring of Low-LG mothers show reduced hippocampal bFGF protein expression at the time of weaning (Figure 10). The neuronal isoforms of bFGF have both autocrine and paracrine effects that promote neuronal proliferation, differentiation and survival (Ray et al., 1993; Wagner et al., 1999; Williams et al., 1994; Aoyagi et al., 1994; Walicke et al., 1988; Delrieu, 2000). Further, an injection of bFGF at the time of birth is associated with increased neuronal survival at the time of weaning, and under in vitro conditions bFGF increases synaptogenesis in hippocampal neurons (Cheng et al., 2002; Li et al., 2002). Liu et al (2000) also demonstrated that as early as the postnatal day 6, the offspring of High-LG have increased levels of BDNF mRNA expression (Figure 32), a finding replicated in a DNA microarray study (Diorio el al., 2000).

Differences in trophic support for differentiated neurons and newly formed synaptic contacts may contribute to reversing the effects of early life adversity

through environmental enrichment during adolescence. An ICV infusion of bFGF+EGF leads to a 40% recovery of total CA1 neurons lost by global ischemia. These neurons, although immature up to 3 months after ischemia, also established functional synaptic inputs and participated in reconstruction of intrahippocampal connections. Furthermore, this trophic support contributed to reducing cognitive deficits associated with ischemic damage (Nakatomi et al., 2002). Indeed, both Koo et al. (2003) and Guilarte et al. (2003) provide evidence that brain-derived neurotrophic factor (BDNF) may play a critical role in reversing the effect of early life adversity. These data are not unlike those of Liu et al. (2000), which showed that as early as day 6 of life, the offspring of High-LG mothers have enhanced levels of hippocampal BDNF mRNA expression by comparison with the offspring of Low-LG mothers (Figure 32).

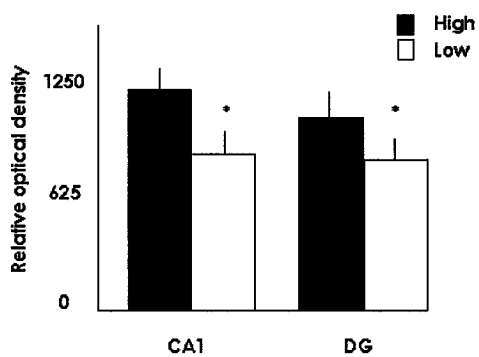


Figure 32. Mean ± SEM hippocampal BDNF mRNA expression (n = 6/group) in 6 day old offspring of High- and Low-LG mothers reared under standard housing conditions ($p < .05$) (Liu et al., 2000).

The hippocampus has the highest expression of BDNF in the mammalian brain (Murer et al., 2001). BDNF supports basal levels of neurogenesis in the dentate gyrus by significantly enhancing the survival of newly generated neurons, and contributes to the enhancement of neurogenesis by dietary restriction in adult mice (Lee, Duan, and Mattson, 2002). BDNF is also critical for synaptic plasticity, particularly in the context of long-term potentiation, and it has been shown that BDNF enhances activity-dependent hippocampal glutamatergic synaptic transmission in both *in vitro* and *in vivo* preparations (Schuman, 1999; McAllister et al., 1997; Schinder and Poo, 2000; Binder et al., 2001; Bramham et al., 2002; Messaoudi et al., 2002; Yamada and Nabeshima, 2003). There is also a strong correlation between hippocampal BDNF mRNA expression and performance in the Morris water maze, contextual fear conditioning and the passive avoidance test (Yamada et al., 2002; Tyler et al., 2002). BDNF mutant mice are impaired in acquisition of spatial learning, and ICV infusion of antisense BDNF oligonucleotides leads to a significant impairment of spatial learning (Linnarsson et al., 1997; Mizuno et al., 2000).

At the cellular level, BDNF phosphorylates the NR1 and NR2B subunits of the NMDAR complex, and upregulates the protein expression of the GluR1 and GluR2/3 subunits of the AMPAR (Suen et al., 1997; Yamada et al., 2002; Jourdi et al., 2003). Moreover, the effects of BDNF on the postsynaptic signal transduction cascade are mediated, in part, by phosphorylation of the NR2B subunit of the NMDA receptor complex at the PSD (Lin et al., 1998; Crozior, Black, and Plummer, 1999). These findings are interesting given the effects of environmental enrichment on glutamate receptor mRNA expression in the offspring of Low-LG mothers. Future

studies into the mechanism driving the ability of environmental enrichment to reverse the effect of reduced maternal care early in life will most certainly involve bFGF and BDNF as major contributors.

6.7. IMMEDIATE EARLY GENES: DRIVERS OF ACTIVITY-DEPENDENT SYNAPTIC PLASTICITY- A central principle of hippocampal synaptic plasticity is that phenotypic changes result from activation of the genome within each cell, such that the appropriate protein substrate is created in order to mediate changes in learning (Bailey, Bartsch and Kandel, 1996). The instruction to initiate activation of the genome and subsequent changes in protein synthesis result from the activity of biochemical transcription factors called immediate early genes (IEGS). IEGs represent the first genetic response to alter synaptic plasticity, and are the mechanism by which short-term events at the cell membrane are coupled to long-term coordinated changes in late onset gene expression (Sheng and Greenberg, 1990; Beckmann and Wilce, 1997; O'Donovan et al., 1999).

Nerve growth factor-induced gene-A (NGFI-A, also known Zif268, Krox-24, TIS8, and Egr-1) is a regulatory IEG associated with experience-dependent synaptic activity, and is expressed in conjunction with stimuli that induce long-term potentiation in hippocampal formation (Worley et al., 1993; Richardson et al., 1992). As early as postnatal day 6 and persisting well into adulthood, the offspring of High-LG mothers show increased NGFI-A mRNA expression in area CA1 of the hippocampal formation, by comparison with the offspring of Low-LG mothers (Figure 33). Expression of NGFI-A is dependent on the activation of the NMDAR,

and NMDA antagonists that block LTP also block the expression of NGFI-A (Cole et al., 1989; Wisden et al., 1990). There is also a positive correlation between NGFI-A mRNA in the CA1 region of the hippocampus and spatial learning performance (Yau et al., 1996).

NGFI-A binding sites have been located on genes encoding synaptophysin and NGFI-A increases hippocampal bFGF expression (Thiel et al., 1994; Biesiada, Razandi and Levin, 1996). NGFI-A is associated with synaptic plasticity (LTP) and learning and is enhanced after environmental enrichment (Dragunow et al., 1996; Yau et al., 1996; Pinaud et al., 2002). In addition, NGFI-A mRNA expression in area CA1 is increased after EE and the major component mediating this effect appears to be social interaction associated with EE (Dahlqvist et al., 1999). Thus, NGFI-A is in a convenient position to link experience with changes in gene expression and synaptic plasticity associated with learning and memory after exposure to environmental enrichment. Interestingly, it has been suggested that NGFI-A expression is driven by exploratory behavior (Wallace et al., 1995). Thus, if the major cognitive differences between the offspring of High- and Low-LG mothers are related to their emotional reactivity to novelty and NGFI-A is expressed in response during exploration of a novel environment, it is possible that this particular activity-dependent transcription factor may drive the influence of maternal care and environmental enrichment on hippocampal development and function.

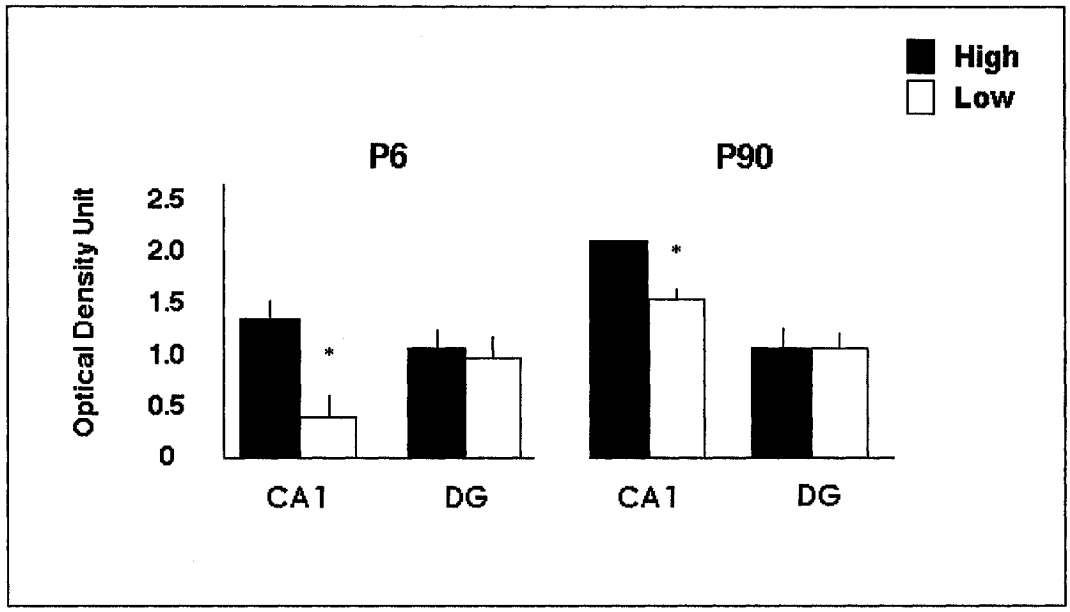


Figure 33. Mean ± SEM hippocampal NGFI-A mRNA expression (n = 6/group) for day 6 and day 90 offspring of High- and Low-LG mothers reared under standard housing conditions. Right panel: representative densitometric micrograph of NGFI-A mRNA expression. (* $p < .05$). (Weaver et al, unpublished data).

Activity regulated cytoskeletal-associated protein (Arc, also known as Arg3.1) is an effector IEG that modifies excitatory postsynaptic function by being selectively targeted to regions of the dendritic arbor in an activity-dependent manner (Steward et al., 1998; Vazdarjanova et al., 2002). Arc associates with the NMDAR complex at the PSD and plays a role in the neurobiological substrate of learning by contributing to structural modification of existing synaptic architecture and, possibly, the formation of new synapses. For example, Arc mRNA increases as a function of learning, and disrupting hippocampal Arc expression impairs LTP and the consolidation of spatial memory (Guzowski et al., 2000, 2001; Kelly and Deadwyler, 2002, 2003). Interestingly, selective translation of Arc into the synaptic density is driven by BDNF, an effect that is dependent on NMDAR activity (Ying et al., 2002). Also, BDNF triggers Arc expression in dentate granule cells and is associated primarily with *in vivo* late-phase LTP (Messaoudi et al., 2002). Thus, the immediate early gene Arc plays a role in stabilizing activity-dependent changes in synaptic efficacy and may participate, along with NGFI-A, in translating maternal care during the early postnatal period and peripubertal environmental enrichment into a direct influence on hippocampal development and function in adulthood.

6.8. ARE THERE COMPENSTORY MECHANISMS ASSOCIATED WITH THE EFFECT OF ENRICHMENT? Reversal and recovery of function have been themes in the literature for quite some time, without revealing the mechanism behind the beneficial effects of enrichment. Evidence for compensatory mechanisms for the functional reversal effect of environmental enrichment come from recovery of

function studies involving naturally occurring and lesion-induced damage to the hippocampal formation. Exposure to environmental enrichment during the peripubertal period reverses the effect of status epilepticus (SE) on performance in the Morris water maze. Enrichment increases neurogenesis and pCREB staining in the dentate gyrus without affecting SE-induced damage throughout the hippocampus (Faverjon et al., 2002; Rutten et al., 2002). Surgical damage to the dorsal hippocampus results in longer latencies to find the platform in both working and reference memory versions of the Morris water maze, and impaired performance in the Hebb-Williams maze (Galani et al., 1997). Environmental enrichment is unable to rescue dorsal hippocampal lesion-induced performance deficits in the novelty test or in the Morris water maze suggesting that the dorsal hippocampus is a primary target for the effects of environmental enrichment. However, enrichment rescues the rats' ability to solve the Hebb-Williams maze, thus raising the possibility that multiple systems are involved in the effects of environmental enrichment on different types of cognitive processing (Galani et al., 1997).

In the Ts65Dn mouse, a model for down syndrome, enrichment ameliorates spatial learning without affecting pyramidal cell structure or cortical circuitry (Martinez-Cue et al., 2002; Dierssen et al., 2003). Environmental enrichment attenuates spatial learning, and trace conditioning deficits but does not rescue the detrimental effect of prenatal alcohol exposure on CA1 pyramidal cell apical and basilar dendrite spine density (Hannigan et al., 1993; Berman et al., 1996). In the R6/2 mouse, a transgenic mouse model of Huntington's disease, limited exposure to environmental enrichment slows the deterioration in motor performance in the

Rotarod test and delays the loss of peristriatal volume without affecting decreased dendritic spine density seen in symptomatic R6/1 mice (Hockly et al., 2002; Spires et al., 2004). Interestingly, these behavioural effects are accompanied by increased BDNF protein expression throughout the hippocampal fomation and striatum (Spires et al., 2004). In Lurcher mice, a mutant characterized by massive degeneration of the cerebellar cortex, enrichment improves motor coordination and water maze learning (Caston et al., 1999). At of yet, the molecular targets mediating recovery of function in this model of neurodegeneration are unknown.

The BSXB mouse is an interesting story; 40-60% of these mice have a developmental anomaly characterized by excessive migration of neurons into the neuron sparse layer I of the neocortex- a phenomenon called neocortical ectopia. However, ectopic mice actually show enhanced spatial reference learning by comparison with their non-ectopic counterparts, a difference that is eliminated under conditions of enriched housing (Boehm et al., 1996; Hoplight et al., 2001). These data provide compelling evidence that higher cortical regions may play an important role in the compensatory effects of environmental enrichment on neural and behavioural development. Considering its executive role as the interface between emotion and cognitive function, the anterior cingulate cortex may be a potential target for future studies concerning the effect of environmental enrichment and maternal care (for a review, see; Allman et al., 2001).

6.9. HUMAN IMPLICATIONS AND THE IMPORTANCE OF HEADSTART-

It is clear that the development and function of the mammalian hippocampal

formation is sensitive to environmental agents, hormonal influences, and sensory stimulation in the form of parent-infant interactions and/or environmental isolation/enrichment. These findings are important when we consider the implications of their influence on abnormal hippocampal function in humans with respect to neurological disorders across the lifespan. For example, there is substantial evidence in favour of the idea that hippocampal NMDARs are involved in the aetiology of schizophrenia (Tsai and Coyle, 2002; Konradi and Heckers 2003). Along with decreased hippocampal glutamate concentration, schizophrenics also show a deficit in NR1 mRNA expression (Gao et al., 2000). Functional brain imaging has demonstrated that schizophrenics perform poorly on hippocampal–dependent learning tasks, and hippocampal NR1 mRNA correlates with cognitive dysfunction (Humphries et al, 1996; Heckers et al., 1998). These findings are paralleled in mice where reduced expression of the NR1 subunit mRNA expression leads to aberrant behaviour mimicking aspects of schizophrenia (Mohn et al., 1999). The relationship between alterations in hippocampal NMDA subunit expression and pathophysiology in human subjects also occurs in Alzheimer's disease. Alzheimer's patients have decreased NR2A and NR2B mRNA, and decreased NR1 and NR2B protein expression that correlate with cognitive function (Sze et al., 2001; Bi and Sze, 2002). Again, findings that are paralleled in the animal literature where it has been shown that antisense knockdown of NR2B mRNA expression leads to cognitive impairments not unlike those exhibited in Alzheimer's disease (Clayton et al., 2002).

Animal studies on the influence of environmental factors for brain and behavioural development have provided an incredible amount of insight into the

neural mechanisms of experience-dependent brain plasticity. However, the findings would be essentially meaningless if we weren't able to bridge the information and extend its relevance toward human populations. In humans, many factors contribute to differences in cognitive development. Biological factors such as neonatal birth complications, and birth weight are associated with cognitive performance in early life (Forfar et al., 1994; Wolke and Meyer, 1999). In addition, environmental factors such as socio-economic status, parenting behaviour and social support also contribute towards cognitive development and the effects of these factors become more important with as the child gets older (Bennett, Bendersky and Lewis, 2002; Butcher et al., 2004). Children with a history of prenatal alcohol exposure demonstrate deficits in language, verbal learning and memory, fine motor performance and visual-motor integration (Mattson et al., 1998, 1999). Further, chronic lead exposure during early development is reported to be associated with cognitive impairments in children (Bellinger et al., 1991; Reiss et al., 1992). There is also relationship between the absence of one parent during childhood and the development of alcoholism, personality disorder, and schizophrenia (Tennant and Bernardi, 1988; Paris, et al., 1994; Furukawa, et al., 1998).

Most of the human research on environmental enrichment focuses on the early postnatal period as being the most critical for intervention strategies. For example, Head start is a federally funded programme for low-income families that provides medical, nutritional and social benefits, and, by pre-school enrichment activity, enables high-risk children to learn more effectively once they reach school. The emphasis of this intervention programme is directed toward children three to five

years of age.. To date, there appear to be no federally recognized programs specifically designed towards providing environmental enrichment for the high-risk adolescent The data from my studies would suggest that adolescence is another critical period in which the effects of adversity early in life on cognitive abilities in adulthood could effectively be reversed through environmental enrichment. Much of the same factors that improve development during early childhood such as improved medical, nutritional and social benefits could also play a role in improving neural development in the adolescent.

6.10. FUTURE DIRECTION- In the studies described in chapter 5, we attempted to elucidate the role of paternal care for offspring cognitive development by removing the father from the family in the monogamous California mouse (*Peromyscus californicus*). The experiment was designed with to test the hypothesis that paternal care influences hippocampal-dependent learning in adult male and female offspring. The findings indicate that, under stable laboratory conditions, paternal care does not affect learning and memory in the male offspring (Figure 26 and 27). However, fathers' presence appears to be important for mediating the effect of neonatal handling on non-spatial learning in male offspring, with no effect in females (Figure 26). Perhaps even more intriguing are the data showing that in non-handled, father-absent families, female offspring show enhanced spatial learning such that they are indistinguishable from animals raised under handled conditions (Figure 27). As a result of the stress of handling and/or the absence of the father in the neonatal environment, perhaps the mother-infant interaction changes such that the course of

cognitive development is altered by a bias in maternal care towards her female offspring. This will be a focus of future studies looking at biparental care effects on offspring cognitive development.

The findings also suggest that the importance of paternal investment may only become clear when environmental demands are such that maternal care is compromised. When parents are required to work for food, the presence of the father influences pup survival (Wright and Brown, 2002). Further, Wright and Brown (2002) also provide evidence that, by increasing his foraging behaviour, the father serves to buffer the mother against environmental stress, thereby allowing her to invest more readily in the offspring. Thus, as another future direction of my research into environmental influences on cognitive development, I am designing a series of experiments based on the method of Wright and Brown (2002) that exploits this allocation of investment strategy as a function of foraging demand, in an effort to further elucidate the effect of bipaternal care during the early postnatal period on offspring neural and behavioural development in adulthood.

6.11. CONCLUDING REMARKS- These studies have provided evidence for reversal at the level of both function (spatial and non-spatial learning), and mechanism (synaptic density and glutamate receptor gene expression). However, it is also clear that much work remains in order to truly elucidate the mechanism by which these effects occur. For example, what drives the effect of enrichment and when does the switch or recovery of function take place? As mentioned, two obvious candidates worthy of future study are the immediate early genes NGFI-A and Arc. Another question concerns individual sensitivity to the effects of environmental enrichment.

Why are the Low-LG offspring more sensitive to enrichment than the High-LG offspring? The difference between High- and Low-LG offspring in hippocampal astrocyte expression will most certainly be a focus of future studies. Below is a schematic representation of the many potential factors and targets that mediate the effects of maternal care and environmental enrichment on hippocampal development and function. This figure serves to remind us of the complexity of the incredible relationship between environment and gene expression, nature and nurture if you will, and their influence on a rat's ability to navigate in the world around it.

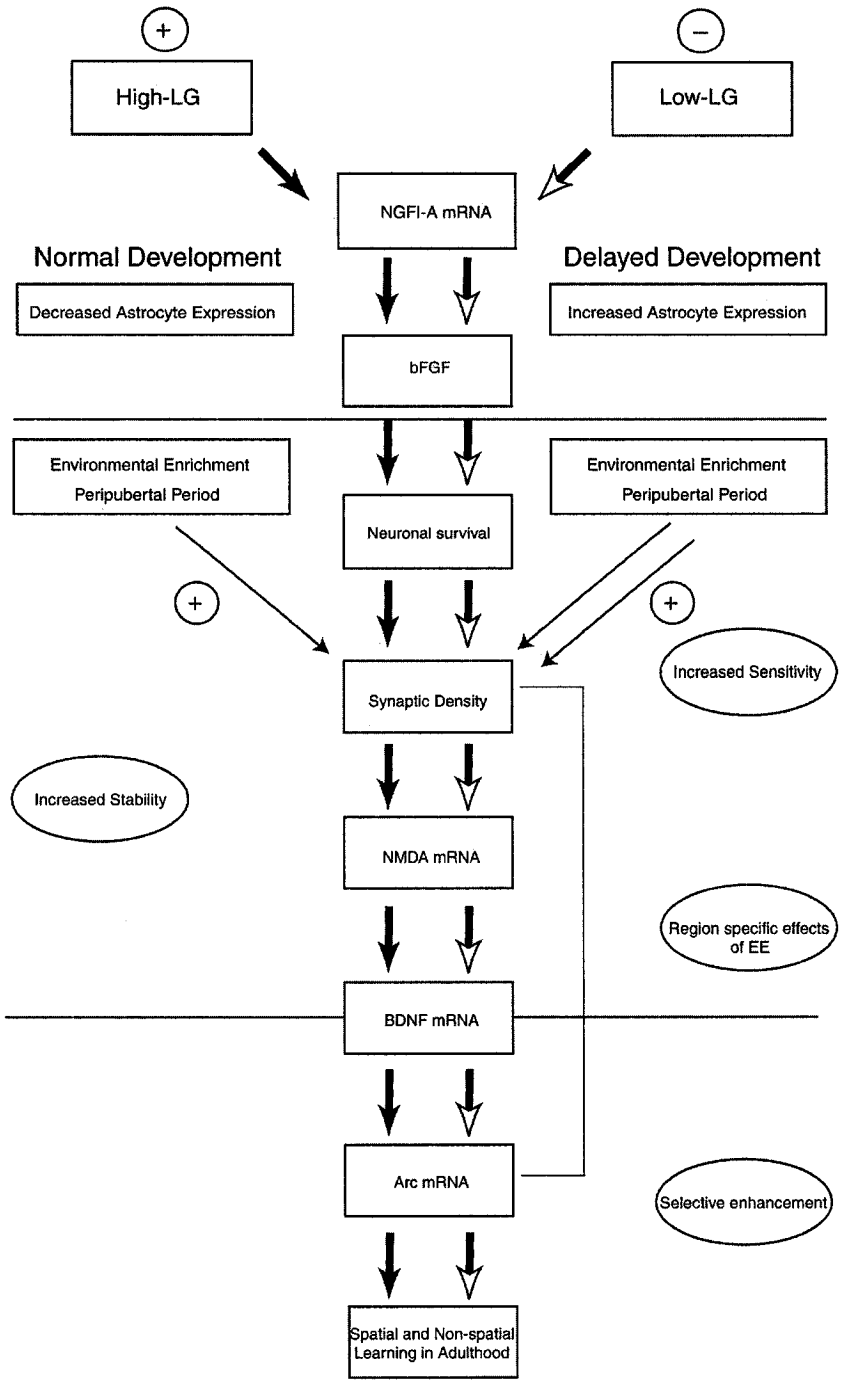


Figure 34. A schematic representation of the potential molecular cascade mediating of the effect of maternal care and environmental enrichment on hippocampal development and cognitive function in adulthood.

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Appendix A. Animal Use Protocol.

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Animal Use Protocol – Research
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Protocol #: 1510
Investigator #:
Approval End Date:
Facility Committee:

☐ Pilot ☐ New Application ☒ Renewal of Protocol #

Title (must match the title of the funding source application):

1. Investigator Data:

Principal Investigator: _____ Office #: _____

Department: _____ Fax#: _____

Address: _____ Email: _____

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: _____ Work #: _____ Emergency #: _____

Name: _____ Work #: _____ Emergency #: _____

3. Funding Source:

External ☒	**Internal** ☐
Source (s):	Source (s):
Peer Reviewed: ☒ YES ☐ NO**	Peer Reviewed: ☐ YES ☐ NO**
Status : ☒ Awarded ☐ Pending	Status: ☐ Awarded ☐ Pending
Funding period:	Funding period:

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed . e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y): or ongoing ☒

Expected Date of Completion of Animal Use (d/m/y): or ongoing ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator:	Date:

Approval Signatures:

Chair, Facility Animal Care Committee:		Date:
University Veterinarian:		Date:
Chair, Ethics Subcommittee(as per UACC policy):		Date:

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