**PRE- AND POST-SYNAPTIC, INPUT-DEPENDENT PLASTICITY
OF THE HIPPOCAMPUS: A STRAIN BY GENDER ANALYSIS**

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by

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Abstract

LER and WR are well known for their morphological differences in the mossy fiber terminal field (MFTF) projection to CA3 as well as developmental differences in this same region. These endogenous differences in the MFTF distribution can be altered through learning-associated environmental input. This study examined learning-associated synaptic changes in adult LER and WR male and female rats over the course in a Morris water maze (MWM) switching task. A second objective was to examine task acquisition in juvenile rats from p16 to p26 and determine how this early experience affected later memory processing. The third objective sought to examine the effect of juvenile pre-training on later MWM acquisition. In both adult groups, morphological changes in the hippocampus were examined. LER males were found to benefit most from juvenile MWM experience. WR males showed some benefit, whereas LER and WR females showed no behavioural improvements due to juvenile MWM experience. Gender differences were only observed in animals given juvenile pre-training whereas no differences were observed between males and females given no juvenile pre-training. These results highlight the importance of strain and gender differences and the effect of juvenile experience on the development of the hippocampus and in the performance of spatial learning tasks.

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List of Abbreviations

ABC – avidin-biotin complex

AMPA_r - α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

DAB – 3,3'-diaminobenzene

DG – dentate gyrus

dH₂O – distilled water

e# - embryonic day...

EC – entorhinal cortex

ETOH - ethanol

GABA_A_r - γ -amino-butyric acid A receptor

GCL – granule cell layer

GCs – granule cells

Glu - glutamate

LER – Long-Evans hooded rat

LTD – long-term depression

LTP – long-term potentiation

MAP2 – microtubule-associated protein 2

MFs – mossy fibers

MFTF – mossy fiber terminal field

MWM – Morris water maze

NGS – normal goat serum

NMDA_r – *N*-methyl-D-aspartate receptor

p# = postnatal day...

PKC – protein kinase C

PBS – phosphate buffered saline

PBS-TX – 0.1% triton-X in phosphate buffered saline

SL – stratum lucidum

SO – stratum oriens

TBS – tris buffered saline

T-TBS – 0.1% triton-X in tris buffered saline

WR – Wistar rat

Introduction

Plasticity

In general, neural plasticity can be described as the brain's inherent ability to change its structure or function with experience (Kalia, 2008). In turn, these changes can facilitate behavioural modification and adaptation (Kalia, 2008). One illustration of neural plasticity is synaptic plasticity, wherein the strength, efficacy or structural arrangement of multiple neuronal synapses is modified through activity-dependent processes (see Citri & Malenka, 2008, for review). Synaptic plasticity that results from environmental experience is thought to be essential for the establishment of neuronal circuits during development and may subserve processes that are critical for both short- and long-term memory storage.

Plasticity and memory

Synaptic plasticity is thought to be the cornerstone of learning and memory and can be subdivided into short-term and long-term processes. Short-term synaptic plasticity refers to the innate capacity for a synapse to modify and adjust its potential for future depolarizations and subsequent signal propagation. Examples of such modifications include post-tetanic potentiation and vesicular location (i.e, movement of vesicles closer to active zone). Long-term synaptic plasticity underscores the establishment of long-term memory and takes the form of long-term potentiation (LTP) or long-term depression (LTD). Functional or structural changes at the synapse last hours to days, and have a long-lasting effect on a neuron's potential for depolarization. LTP is correlated with an

increase in size and number of dendritic spines whereas LTD is correlated with a decrease in size of dendritic spines (Bock & Braun, 1999; see Sur & Rubenstein, 2005, for review). These changes are thought to play a key step in not only learning and memory function but also brain development.

Structural or functional synaptic plasticity requires local changes in Ca^{2+} concentrations in dendritic spines, most often occurring through the activation of the *N*-methyl-D-aspartate receptor (NMDAr), a glutamatergic ionotropic receptor (Bock & Braun, 1999; Citri & Malenka, 2008; Rabacchi et al., 1992). NMDAr activation directly elevates intracellular Ca^{2+} concentrations postsynaptically (Liu et al., 2004) and is known to be a critical component for initiating plasticity at synapses (Nicoll, 2003). NMDAr activation occurs when there is sufficient postsynaptic depolarization typically due to glutamate binding to α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAr), which in turn results in the removal of a Mg^{2+} block from the NMDA channel. Removal of the Mg^{2+} block allows for extracellular Ca^{2+} to enter the postsynaptic cell. Activation of NMDAr leads to a potentiation that decays in less than one hour (short-term plasticity) suggesting the necessity for downstream factors to establish long-term synaptic alterations. These factors include activation of protein kinase C (PKC), Ca^{2+} /calmodulin-dependent kinase II, phospholipase A_2 and other calcium-dependent processes (Sabatini et al., 2001) all of which are thought to be critical for the maintenance of long-term synaptic changes. Thus, it appears that synaptic plasticity is underscored by strict regulation of intracellular Ca^{2+} concentrations within pre- and postsynaptic terminals (see Kalia, 2008, for review).

Anatomical analysis of the frequency and structure of spines suggests that a number of input-dependent processes produce an increase in the size of the postsynaptic density (see Edwards, 1995, for review). Outgrowth of spines may be responsible for persistent functional changes resulting from synaptic potentiation (Chen et al., 2004). Early studies (Volkmar and Greenough, 1972; Greenough et al., 1973; 1979 Greenough and Volkmar, 1973; Globus et al., 1973) found that exposing animals to enriched environments was associated with an increase in the numbers of dendritic spines. Other structural changes may include division of already present spines (Carlin and Siekevitz, 1983) or fine structural changes of the spine head (Boothe et al., 1979). An increase in the number of spines has been associated with synapses showing increased efficacy for information storage (Geinisman, 1993). In hippocampal slices, LTP induction in CA1 synapses leads to the appearance of new postsynaptic dendritic spines while no spine outgrowth occurred in areas where potentiation was chemically blocked (Engert and Bonhoeffer, 1999). Hippocampal CA1 neurons expressing green fluorescent protein demonstrated that high frequency stimulation enhanced postsynaptic spine growth that was long lasting and localized to the stimulated dendrite (Maletic-Savatic et al., 1999).

An additional structural alteration underlying long-term synaptic enhancement is the remodeling of presynaptic terminals (Cantalops et al., 2000). Previous work found that water maze overtraining to find a hidden platform increased the density of the mossy fiber terminal field (MFTF) in the CA3 stratum oriens (SO) of adult Wistar rats (WR), which is an area not normally innervated by mossy fibers (MFs) in this particular strain (Ramírez-Amaya et al., 1999; 2001; Holahan et al., 2006; Rekart, et al., 2007a and b).

This suggests that axonal growth may mediate persistent changes in long-term memory storage (Ramírez-Amaya et al., 2001).

Development and plasticity

Normal development consists of anatomical and connectivity-based changes in the structures of the entire body, including the central nervous system. The developing nervous system requires large amounts of pruning and remodeling in order to achieve its final, adult configuration (Kantor & Kolodkin, 2003). These connectivity-based changes are a result of the brain's intrinsic plasticity, which is determined by innate genetic differences and responses to external stimuli (see Sur & Rubenstein, 2005, for review).

The organization of neurons throughout the brain begins prenatally by radial migration of neuronal precursors from the ventricular zone (see Caviness & Takahashi, 1995, for review). Following migration, neurons establish connections through the sprouting of dendrites and axons and the seeking out of synaptic contacts. This process of forming stable structural connections is followed by neuronal pruning and programmed cell death (see Meier et al, 2000, for review), wherein neurons are selected for or against in terms of connection strength, propriety and efficiency (Cowan et al., 1984; Purves & Lichtman, 1980). Approximately 50% of postmitotic neurons viewed during development appear to survive into adulthood (Low & Cheng, 2000). Pruning requires significant microtubule assembly and disassembly in order to achieve the appropriate connection pattern (Kantor & Kolodkin, 2003). These final stages of pruning are organized through activity-dependent processes (see Luo & O'Leary, 2005; Sur & Rubenstein, 2005for review) and breakdown of the cytoskeletal structure required for

pruning is postulated to be a Ca^{2+} -dependent process (Raff et al., 2002). In addition to pruning, the process of apoptosis is determined not only by genetic programming but also results from complex interactions between genes and the environment, mediated by activity-dependent processes (see Kalia, 2008 for review). Thus the structural growth and development of the brain, a sequence based on synaptic plasticity, similar to that which occurs in learning and memory, consists of interactions between genetically programmed changes as well as input from external stimuli.

Hippocampal plasticity – Anatomy

The hippocampus has long been studied for its prominent role in learning and memory. Specifically, the hippocampus demonstrates remodeling and synaptic plasticity that may be pivotal for the establishment and maintenance of long-term memory.

Anatomically, the hippocampus can be divided into four principal regions: the dentate gyrus (DG), the hippocampus proper, which itself is made up of CA1, CA2 and CA3 subregions, the subicular complex, and the entorhinal cortex (EC) (Amaral & Witter, 1989). From a general perspective, these hippocampal fields are connected through unidirectional excitatory projections (Amaral & Witter, 1986), though some subregions do display internal connectivity, interhemispheric connections, unidirectional inhibitory connections, feedback loops and numerous other patterns of connectivity. The present review focuses on the DG and its connections with the CA3 region.

The DG is composed of three layers, the polymorphic layer, the molecular layer and the granular layer (Gaarskjaer, 1986). The polymorphic layer contains a variety of neuronal cell types (Amaral & Witter, 1989). The molecular layer consists of the

dendritic arbors of the granule cells (GCs) (Gaarskjaer, 1986) and receives innervation from the entorhinal cortex via the perforant path (Amaral & Witter, 1989). The granular layer is formed primarily of GCs packed into two U-shaped, interlocking sheets (Heimrich & Frotscher, 1991). The number of GCs in the dentate gyrus is approximately 1.2×10^6 (Gaarskjaer, 1978). Typically, GCs have little cytoplasm and a spherical cell body containing a 10-15 μm diameter nucleus (Gaarskjaer, 1986; Heimrich & Frotscher, 1991). GCs demonstrate bipolarity; their axons, the mossy fibers (MFs) originate from the basal pole, and the dendritic arborizations of the GCs are concentrated at the apical pole (Heimrich & Frotscher, 1991). The DG is one of the few neural structures to exhibit postnatal neurogenesis throughout adulthood in rats as well as in humans (Mizuhashi et al., 2001). New GCs are continually being generated from progenitor cells in the hilus, though the number of GCs remains relatively constant throughout adulthood, regardless of the age of the individual in question (Henze et al., 2000). The pattern of neurogenesis in the DG GCs proceeds from lateral to medial and from superficial to deep (Gaarskjaer, 1986). Thus, GCs located more laterally are older and those located more medially are younger. GC proliferation can be affected by a variety of factors, including exposure to novel environments and a number of stressors, which increase and decrease GC proliferation, respectively (Henze et al., 2000; Akman et al., 2004).

MFs are described as the thin axon collaterals extending from dentate GCs to innervate the principal neurons of the CA3 region. MFs typically measure 0.1-1.7 μm in diameter (Gaarskjaer, 1986) and provide the main excitatory innervation to the hippocampal CA3 region (Bliss et al., 1974; Gaarskjaer 1978; West et al., 1980; West, 1983; Amaral & Witter, 1989; Henze et al., 2000). MFs collateralize in the polymorphic

layer of the DG, then enter the CA3 field where they form synapses with the CA3 pyramidal cell apical dendrites (Amaral & Witter, 1989). MFs rarely have axoaxonic connections with other MFs (Amaral & Dent, 1981). MF axonal boutons are large in size, measuring 4-10µm in diameter and contain very high levels of zinc (Henze et al., 2000). One MF axon innervates, on average, fourteen CA3 pyramidal cells (Henze et al., 2000). MF boutons are enormous in size (Bliss et al., 1974) and contain a large density of synaptic vesicles encapsulating various neurotransmitters (Staubli, et al., 1990) and zinc (Wenzel et al., 1997). Glutamate (Glu) is viewed as the main neurotransmitter released at MF synapses (Gaarskjaer, 1986).

The CA3 region is lateral to the DG and is made up of large pyramidal cells, which are contacted by the MFs of the GCs (Gaarskjaer, 1986). The CA3 region also receives synaptic input from the EC via the perforant path (Gaarskjaer, 1978). The CA3 region has a large amount of intrinsic connectivity, demonstrated by large associational axonal projections from CA3 pyramidal cells onto other CA3 pyramidal cells (Amaral & Witter, 1989). The axons of the CA3 pyramidal cells also provide the main excitatory input to the CA1 region; these axons are referred to as the Schaffer collaterals (Amaral & Witter, 1989). The CA3 region can be subdivided into the stratum radiatum (SR), the stratum lucidum (SL) and the stratum oriens (SO). The apical dendrites in the SL receive the majority of the MF innervation from the DG (Amaral & Witter, 1989) while the basal dendrites in the SO receive varying amounts of synaptic inputs from GCs.

MFs are easily viewed using the Timm's staining technique, which views axon terminals containing Zn. Zn is known to have modulatory effects on both voltage- and ligand-gated channels, demonstrating specific inhibitory effects on NMDAr (Deizler &

Leonard, 1997; Wenzel et al., 1997) and on γ -aminobutyric acid A receptor (GABA_AR) (Wenzel et al., 1997). Zn-positive staining is a ubiquitous technique for the microscopic visualization of the mossy fiber terminal field (MFTF).

Hippocampal plasticity – Spatial learning

The hippocampus has a well-demonstrated role in mediating spatial information processing (Ramos, 2000; see Bird & Burgess, 2008 for review) and in particular, the DG-CA3 pathway shows activity-dependent, long-term structural modifications that may play a pivotal role in the learning and memory process.

Spatial learning is highly influenced by the distribution of the MF terminal field on the apical and basal dendrites of the CA3 pyramidal cells (Cantalupo & Routtenberg, 1999; Schöpke et al., 1991; Holahan et al., 2006). There is a strong positive correlation between a larger MF terminal field distribution and superior performance in the Morris water maze (MWM) (Roskoden et al., 2005; Holahan et al., 2006), as well as Y-maze discrimination, simple radial-maze learning and habituation and exploration in open field testing (Crusio & Schwegler, 1987; Jacobs et al., 1990; Schwegler et al., 1990; Schwegler & Crusio, 1995). The size and extent of the MF projection has been demonstrated to facilitate spatial learning, working memory and memory consolidation (Crusio & Schwegler, 1987). Disrupting the function of MFs can negatively impact spatial learning, in particular on the MWM task. Lassalle, et al., 2000, found that mice with selectively inactivated MFs were impaired in the MWM task if inactivation occurred before training. Thus, the MFs play an important role in spatial processing during the MWM task.

Hippocampal development

The MFs provide the main excitatory input from the GCs of the DG onto CA3 pyramidal cells (Bliss et al., 1973; Gaarskjaer, 1986; Kantor & Kolodkin, 2003). It is thought that MFs demonstrate activity-dependent synaptic pruning during development (Laurberg & Zimmer, 1981) as well as during learning and memory formation later on in life (Hastings & Gould, 1999).

The formation of the rat DG begins at embryonic day 16 (e16) (Schwab et al., 2000), although the first GCs are formed in the ventricular zone between e14 and e17 (Gaarskjaer, 1985). However, 80% of GCs are formed postnatally (Gaarskjaer, 1985). Synaptic connections in the DG increase at the greatest rate from postnatal day 4 (p4) to p11, then taper off until p25 (Cotman et al., 1973). Their development is determined by GABA- and Glu-mediated processes (Wang & Kriegstein, 2009). By p25, synaptic densities in the DG have attained their adult configuration, which is similarly observed in other brain areas including the cerebellum (Crain et al., 1973).

NMDAr activity is critically linked to the normal development of hippocampal circuitry. *In vitro*, chronic NMDAr blockade from p0 to p14 leads to an increase in synapse number and complexity in the GCs of the DG (Cantallos & Routtenberg, 1999) and other hippocampal subregions (Rabacchi et al., 1992). Thus, it is hypothesized that synaptic formation during development is determined by NMDAr-independent processes while synaptic pruning and elimination is determined by NMDAr-dependent processes in the hippocampus (Lüthi et al., 2001). Note the similarity to the plasticity processes as described for learning and memory above.

In Long-Evans hooded rats (LER), MFs are present on p1 in the stratum lucidum

(Amaral & Dent, 1981). By p10, the MFs appear to have a configuration similar to their adult morphology, with the dendritic spines in CA3 having made connections with the MFs (Amaral & Dent, 1981; Gaarskjaer, 1985). Between p4 and p11, existing MFs help guide newly formed MFs to their target location (Nguyen et al., 1996). By p14, the size and density of the MFs increases, showing a resemblance to the adult morphology (Amaral & Dent, 1981). Thus, most of the MF growth is experienced during the first two weeks of life in LER (Amaral & Dent, 1981). Finally, by p21, most of the functional maturation of the MFs has occurred, though differences over the course of adulthood are often observed (Amaral & Dent, 1981; Henze et al., 2000). The MF boutons appear fully matured by p28, showing increased size, and differ very little from MF boutons seen at p420 (Amaral & Dent, 1981). MF development appears to parallel the development of the DG GCs, in both space and time (Amaral & Dent, 1981). Immature GCs extend MFs into the CA3 region of the hippocampus anywhere from 4 to 10 days following mitosis (Hastings & Gould, 1999), and there is a correlation between the arrangement of the MFs and the arrangement of the CA3 pyramidal cells (Gaarskjaer, 1986).

It has been proposed that the size of the MFTF is determined solely by genetic factors and is not influenced by environmental factors early in life (Gaarskjaer, 1986; Schwegler et al., 1990). However, neonatal handling or maternal separation may result in a significant reduction in MF axon development in CA3 as well as mild impairment of hippocampal-dependent spatial learning (McNamara et al., 2002). Furthermore, stress and environmental enrichment can affect MFTF development. Thus, although MF development appears to be almost exclusively determined by genetic influences, early-life experience and stress can potentially have an effect on their outgrowth and

subsequent innervation patterns. Since neurogenesis occurs in the DG not only prenatally but postnatally and throughout adulthood, there is constant synaptic structural plasticity occurring between the GCs of the DG and the pyramidal cells of CA3 (Mizuhashi et al., 2001).

Spatial learning – The Morris water maze

The study of spatial learning in both rodents and humans has long involved the examination of the hippocampus (see Burgess, 2008 for review). Spatial learning consists of the ability to learn and remember the relationship between the location of different objects in an environment in a three-dimensional coordinate system. Spatial learning is typically examined in terms of allocentric or egocentric cues, wherein allocentric cues typically are in reference to the environment and egocentric cues are in reference to oneself. There are a variety of mazes used for the study of spatial learning in rodents, including radial arm maze, T-maze and Y-maze. Here, the focus lies on the Morris Water maze, one of the most widely used tools in the study of spatial learning in rodents.

The MWM typically examines the ability of an animal to remember the location of a platform in a pool of water, using external cues for guidance and spatial navigation. This task can be modified in a variety of ways, including submerging or removing the platform as well as varying the presence, absence or number of intra- and extramaze cues. Further, the water within the MWM may also be rendered opaque in order for a platform location to be determined solely by memory and not by the visualization of the platform itself.

Performance in the MWM is influenced by a combination of the intrinsic

characteristics of the animals being tested (including strain and gender) and by differences in apparatus and training procedure. A general review of the Morris water maze (MWM) in terms of age, strain and gender differences as well as a complete description of the application and procedure involved in MWM testing is described in D'Hooze & De Deyn, 2001.

Pups and adult rats use a variety of cues in order to navigate in a MWM, including direction and distance cues (Akers et al., 2007). Rats are able to swim at p16, but require a mature hippocampus in order to accurately succeed in the MWM task (Rossier & Schenk, 2003). Rats can demonstrate adequate spatial navigation skills as early as p17 despite their relatively immature hippocampi (Carman & Mactutus, 2001). Vision plays a large role in spatial navigation in rats, whereas olfaction and audition play more minor roles (Rossier & Schenk, 2003); however, if vision is impaired, audition and olfaction are more heavily relied on for spatial navigation. The principle of interocular equivalence dictates that neural networks from each eye must converge on neural structures mediating memory formation in order for visual cues to be properly integrated (Rudy & Paylor, 1987). Rats do not demonstrate interocular equivalence before p28, thus may be unable to integrate visual cues until this age (Rossier & Schenk, 2003). It is speculated that cues in multiple modalities may be important when training rats younger than p28.

Early-life experience appears to have beneficial effects on learning in spatial memory tasks. Specifically, rats trained and tested in a radial arm maze from p16 to p20 performed significantly better than age-matched, untrained controls at p22, 23, 24 and 25 (Carman & Mactutus, 2001). Though pups trained and tested in spatial memory tasks do

not necessarily appear to show learning acquisition, due to incomplete hippocampal development, they do demonstrate beneficial spatial learning strategies when tested later on (Carman & Mactutus, 2001).

LER & WR hippocampal differences

Structure

Similar to the albino Wistar rat (WR) strain, the CFY strain, also an albino rat, has been shown to differ from LER rats (pigmented strain) in their innate number of granule cells (Schwegler & Crusio, 1995). Holahan et al., 2007, examined the differences in adult LER males in comparison to WR counterparts and found that the suprapyramidal MFTF in LER extends into distal SO, whereas this pattern of innervation is not seen in WR. WR instead show MFTF staining exclusively into the SL. Their patterns of MFTF innervation were reversed through NMDAr blockade; LER animals treated with the NMDAr antagonist CPP from p17 to p20 showed decreased staining of the SO at p40 as compared to saline-treated LER (Holahan et al., 2007). In contrast, p40 WR treated with CPP from p17 to p20 showed increased MFTF staining in SO as compared to saline-treated WR (Holahan et al., 2007). Therefore, the outgrowth and pruning of the MFTF in the LER and WR in the CA3 region is determined by activity-dependent processes specifically involving NMDAr. These results were confirmed using Timm's staining for the identification of Zn-positive MFTF, synaptophysin for the identification of presynaptic terminals and Tau1 as an axonal marker (Holahan et al., 2007). Adult WR were shown to develop a MFTF innervation into the SO in CA3 when given MWM overtraining (Holahan et al., 2006). Therefore, although both WR and LER show

inherent, presumably genetic differences in their MFTF distribution, which are mediated by activity-dependent processes, these differences may be “rescued” through behavioural and pharmacological intervention.

Development

The differences in adult patterns of innervation in the hippocampus seen in the LER and WR strains were examined in terms of their developmental time course. By p15, both LER and WR showed increased Timm’s staining in SL, indicating MF innervation of this CA3 subfield (Holahan et al., 2007). By p18, both strains also showed distinct Timm’s staining in the SP and the dorsal portion of SO of CA3 (Holahan et al., 2007). By p21, both strains showed the same innervation pattern of the hippocampal CA3 region, with distinct Zn-positive MFTF staining throughout the entire CA3 region, which was similar to the pattern of staining seen at p18 (Holahan et al., 2007). This finding was also confirmed through the examination of synaptophysin staining which showed increased numbers of presynaptic terminals in SO in both strains (Holahan et al., 2007). However, at p24, WR and LER differed in their pattern of CA3 innervation; LER retained the hippocampal morphology as observed on p21, whereas WR no longer showed any detectable Zn-positive staining or synaptophysin staining in SO (Holahan et al., 2007). WR also showed increased staining in SL, which is a pattern thought to be indicative of the retraction of the MFs from SO to SL in the WR (Holahan et al., 2007). This MF innervation of CA3 in both strains at p24 was maintained to p33 as well as into adulthood (Holahan et al., 2007). Thus, although LER and WR share similar developmental patterns of MFTF up until p21, after p21, the WR pattern of CA3 MF

innervation shifts, resulting in retraction of their MFs from SO to SL.

Spatial Memory

Albino rat strains perform differently from non-albino rat strains in terms of MWM spatial learning (for a review, see D'Hooge & De Deyn, 2001). Holahan et al., 2006 examined MWM task acquisition in both the LER and WR strains. In a visible platform MWM task, both strains learned to swim to and mount the platform at a similar rate, suggesting that eye pigmentation and visual acuity did not drastically affect acquisition of this task (Holahan et al., 2006). However, when the platform was moved to a new location on the second day, WR demonstrated a longer latency to reach the new platform location, increased old platform location crossings, as well as an increased amount of time spent swimming around the perimeter of the pool compared with the LER strain (Holahan et al., 2006). Thus, WR show inferior performance, as compared to the LER strain, in the MWM task when the platform is moved, perhaps showing a deficit in learning flexibility.

Male & female hippocampal sexual dimorphisms

Structure

The dentate gyrus granule cell layer (GCL) shows sexual dimorphism in rats. In general, it appears that male rodents have a larger volume of GCs in the DG, although this difference can differ between strains and with hormone treatment (Tabibnia et al., 1999; Hajszan et al., 2007). Females treated with testosterone and non-treated males show a thicker GCL as compared to non-testosterone-treated females (Roof & Havens, 1992). As well, it appears that males and testosterone-treated females show hemisphere

asymmetry, with the width of the GCL appearing thicker in the right as compared to the left hemisphere (Roof & Havens, 1992). The sexual dimorphisms in the GCL appear in the absence of any differences observed in pyramidal cell layer morphology or thickness (Roof & Havens, 1992). These morphological changes in the hippocampus appear exclusively hormone-related, considering the similar morphology of testosterone-treated female and male rats. Also, it appears that spine and synapse number in the hippocampus are also sensitive to hormonal state of females; synapse and spine number are positively correlated with levels of circulating estrogen (Woolley & McEwen, 1994; Warren & Juraska 1997). When females are in proestrus, they demonstrate the highest levels of synapse and spine number. When females are in estrus, they demonstrate the lowest levels of synapse and spine number.

Function

LTP in the Schaffer collateral pathway has been reported to vary in terms of gender, time of day and phase of estrus cycle (in females) (Warren et al., 1995). All groups, regardless of gender or estrus phase for females, had an increased response to LTP stimulation in the morning as compared to the afternoon (Warren et al., 1995). In the afternoon however, females in proestrus had greater responses to LTP stimulation as compared to the estrus and diestrus groups (Warren & Juraska, 1995). Thus, it is important to consider not only the time of day for any sort of examination of the hippocampus but also phase in the estrus cycle in female rats.

Spatial memory

In a meta-analysis by Jonasson, 2005, it was concluded that male rats demonstrated a consistent advantage in standard MWM protocols across a number of rat strains. In a variety of spatial maze types, male laboratory rats have been observed to make significantly fewer errors than females, although this difference was decreased in experiments using pre-training protocols (Jacobs et al., 1990). Females also show increased activity in open-field testing, perhaps explaining their increased error rate in spatial memory tasks (Jacobs et al., 1990; Silva-Gómez et al., 2003). Age may also have an impact on sex differences, considering male and female LER tested at 180 days of age showed no differences in spatial memory in a traditional MWM (Bucci et al., 1995). Further examination of male and female differences, with focus on the female estrus cycle, demonstrated, regardless of the hormonal state of the female, there were no differences between the sexes in terms of MWM acquisition (Warren & Juraska, 1997). When the females were determined as being either in estrus or proestrus, females in the estrus phase outperformed those in the proestrus phase (Warren & Juraska, 1997). It is presumed that lower levels of estrogen benefit spatial learning abilities. However, these results are not necessarily the most widely accepted. Korol (2004) examined the modulation of memory function by estrogen and concluded that estrogen promotes hippocampal strategies for solving spatial tasks. Thus, it is presumed that higher levels of estrogen should facilitate spatial tasks involving the hippocampal formation. In short, there are conflicting results of the effects of hormonal state in females on spatial memory performance. Thus, it is very important to consider the female's hormonal state when examining spatial memory task learning and acquisition.

Environmental enrichment

Environmental enrichment is loosely defined as a combination of complex inanimate and social stimulation (van Praag et al., 2000). Standard laboratory housing for rats often consists of single or double-housing with minimal contact with complex objects save for nesting material and water bottles. Food is often provided *ad libitum*, which results in constant access to food and therefore no need for complex foraging behaviours. Environmental enrichment typically consists of social housing with two or more individuals in a cage, as well as access to complex inanimate objects. These objects could include plastic tubes, running wheels or larger cages with more nesting material as well as access to novel toys or objects. Foraging behaviour can also be induced in such an environment. Standard environmental enrichment procedures can vary in duration lasting less than an hour a day to constant exposure. Enriched environments at an early age can result in improved performance in spatial learning tasks (Tees, 1999; Leggio et al., 2005), increased dendritic complexity in the hippocampus (Altschuler, 1979; Pham et al., 1999; Leggio et al., 2005) and increased numbers of new neurons in the DG (Kempermann et al., 1997). These effects were found to be independent of social housing (Williams et al., 2001) and were found to be less drastic when compared to animals exposed to environmental enrichment as adults (Fiala et al., 1978)

Purpose of this experiment

Given the previously described developmental differences between the LER and WR strains, as well as the differences observed in the adults on the MWM task, the goal

of the present thesis was to examine MWM task acquisition in pups and adults across both LER and WR strains as well as between genders and the subsequent effect of learning on MFTF structural plasticity. Juvenile experience was examined to determine whether it had an effect on, not only later MWM task acquisition as adults, but also the neuroanatomical correlates of these behavioural changes in terms of differing MF and CA3 morphology. Juvenile experience was considered an analog of not only MWM pre-training but also juvenile environmental enrichment.

Given the previously described superior performance of LER males as compared to WR males in the MWM task, it was hypothesized that the LER males would show superior performance in a modified version of this task and would benefit the most from juvenile pre-training. This superior performance should translate into increased amounts of learning-associated changes in the MFTF morphology in the CA3 region of the hippocampus. The superior performance of the LER strain should also be applicable when comparing LER and WR females. This study also examined potential gender differences within both strains.

Experiment 1 – Adults with no juvenile MWM experience

Male and female LER and WR adults were trained in the MWM task for 5 consecutive days. They were examined for learning-associated synaptic changes in terms of dendritic and MFTF morphology in area CA3 of the hippocampus. Comparisons were made between strains, genders as well as within females for phase in the estrous cycle.

Experiment 2 – Adults with juvenile MWM experience

Male and female LER and WR pups were trained in the MWM task from p16 to p26. Once matured, these pups were again tested in the MWM, for 5 consecutive days, in order to examine the effect of juvenile experience on MWM performance. Following training, adults were examined in terms of dendritic and MFTF morphology in the CA3. Comparisons were made between strains and genders as well as within females for phase in the estrus cycle. Comparisons were also made between animals within strain and gender groups either with or without juvenile experience.

Materials and Methods

Subjects

Pregnant Long- Evans hooded (LER; n=7) and Wistar (WR; n=7) female rats were purchased from Charles River Laboratories and singly housed. All pups were used (20 LER males, 19 LER females, 18 WR males and 25 WR females) for either Experiment 1 or Experiment 2. The day the pups were born was marked as P0. Pups were weaned at 21 days of age (p21). When they were weaned, rats were housed in groups of two or three (males and females in separate cages) for the remainder of the experiment. All animals were housed in a temperature controlled vivarium in polycarbonate cages (48 x 26 x 20 cm³) with a 12 hour light-dark cycle (lights on 8h00, lights off 20h00). Food and water were provided *ad libitum*. All experiments were conducted in accordance with the Canadian Council on Animal Care guidelines and an approved Carleton University Animal Care Committee protocol (see Appendix A).

Apparatus – Morris water maze

Different pool and platform sizes were used for the adults and juveniles for the Morris water maze tasks (see Experiment 1 and 2 water maze sections below). The pools were opaque, white and made from polypropylene. The water in the pools was cloudy due to the addition of powdered, nontoxic poster paint. Water temperature remained approximately constant at 21°C throughout the behavioural experiments. In both pools, the platform was submerged 2.0 cm below the water surface. After each training trial, animals were dried off and placed into a heated holding cage. Visual cues such as posters

and geometric shapes were placed on the walls at distances of 43cm, 188cm, 54cm and 104cm and ceiling at a distance of 195cm from the pool. The experimenter remained in the same position throughout all trials. Both adult and juvenile water maze testing was conducted in the same testing room. All rat movements within the pool were tracked using the Smart Tracking System (San Diego Instruments, San Diego, CA) or the 2100 Plus tracking system (HVS Image, Buckingham, UK). Following the final trial on each day, every animal was dried with a towel and placed in another holding cage on a heating pad in the housing room for 10 to 15 minutes after which it was returned to its home cage.

Immunohistochemistry – General procedure

Sectioning

All brains were hemisected and right hemispheres were sectioned at 20 μ m on a Leica CM1900 cryostat (Weztler, Germany) at a temperature of -20°C. Sections were taken exclusively from the right dorsal hippocampus. Sections were stored in a 0.1% sodium azide solution in 0.1M phosphate buffered saline (PBS) at 4°C. For a schematic representation of the hippocampus, see Appendix B.

Immunofluorescence – Synaptophysin and MAP2

Sections from the right dorsal hippocampus only were collected. Sections were single- or double-labeled for synaptophysin and/or microtubule-associated protein 2 (MAP2). Synaptophysin is a synaptic vesicle protein used as a marker for synaptic

density (Valtorta et al., 2004). MAP2 is described as a ubiquitous cytoskeletal neuronal protein that maintains microtubule stability and is specifically found in dendritic arbours of neurons (Garner et al., 1988). Sections were stained using fluorescently-labeled secondaries (for double labeling) or biotinylated secondaries for single-labeled sections (described in next section). All staining procedures were conducted at room temperature. For the fluorescent procedure, sections were washed in 0.01M tris-buffered saline (TBS) for 15 minutes. This was followed by a blocking step in 1% filtered normal goat serum (NGS) in 0.1% triton-TBS (T-TBS) for 1 hour. Blocking was conducted to prevent non-specific binding of the primary antibody through the removal of reactive substances within the tissue for the particular host animal in which the primary antibody was made. The sections were then incubated in primary antibodies (synaptophysin 1:500; MAP2 1:1000; Millipore/ Chemicon, Billerica, MA, USA) overnight at room temperature in 0.01% T-TBS. The following day, the tissue was washed twice in 0.01M TBS for 15 minutes. This was followed by a 2-hour incubation period in the secondary antibodies (1:500 goat anti-rabbit, for synaptophysin; 1:500 goat anti-mouse for MAP2; (Molecular Probes, Invitrogen, Burlington, ON, Canada). Following this, sections were washed for in 0.01M TBS for 15 minutes then mounted on glass slides and coverslipped using a DAPI hardset-mounting medium (Vector laboratories, Burlingame, CA, USA) and stored protected from light at 4°C. Staining was viewed using a Nikon CF160 D-Eclipse Confocal microscope and EZ C1 3.20 software for image capture (Nikon Canada, Mississauga, Ontario, Canada).

Immunohistochemistry for synaptophysin and MAP2

Sections from the right dorsal hippocampus were taken as described above. Sections were stained for either synaptophysin or MAP2. All staining procedures were conducted at room temperature. Sections were washed three times in 0.01 M phosphate buffered saline with 0.1% triton-X (PBS-TX) for 5 minutes per wash. This was followed by blocking in 0.3% hydrogen peroxide (H_2O_2) in 0.01M PBS-TX for 15 minutes at room temperature to minimize background staining. Hydrogen peroxide was used in order to quench the tissues and inactivate any endogenous peroxidases. The sections were then washed three times in 0.01M PBS-TX for 5 minutes each wash. Sections were blocked in a solution of 3% filtered NGS in 0.01M PBS-TX for 30 minutes. This was followed by an incubation in the primary antibody (1:50,000 synaptophysin; 1:100,000 MAP2; Millipore, Billerica, MA, USA) in a 1% NGS, 0.01M PBS-TX solution overnight. The next day, sections were washed three times in 0.01M PBS-TX for 10 minutes each. Next, the tissue was incubated in the secondary antibody for 2 hours (1:500 goat anti-rabbit for synaptophysin; 1:500 goat anti-mouse for MAP2; Vector laboratories, Burlingame, CA, USA) in a 1% filtered NGS, 0.01M PBS-TX solution. The sections were then washed three times in 0.01M PBS-TX for 10 minutes each wash. This was followed by an incubation in an avidin-biotin complex (ABC Elite Kit; Vector laboratories, Burlingame, CA, USA) in 0.01M PBS-TX for 1 hour. The tissue was washed three times in 0.01M PBS for 5 minutes each. Tissue was then developed in 0.5% 3,3'-diaminobenzene (DAB) with 1% cobalt chloride, 1% nickel ammonium sulfate and 0.01% H_2O_2 in 0.01M PBS until sections were fully developed. Sections were determined to be fully developed

when staining appeared dark in colour and specific to synaptic terminals. Developing took approximately 5 minutes. Sections were then washed in 0.01M PBS for five minutes and were stored in 0.01M PBS overnight at 4°C for mounting. Sections were mounted onto slides and allowed to dry.

Mounted sections were dehydrated in serial solutions of 70%, 95% and 100% ETOH for 1 minute each. They were defatted in Clearene® (Surgipath Canada, Inc., Winnipeg, MB, Canada) for at least 10 minutes then coverslipped with Clarion™ (Sigma-Aldrich, St. Louis, MO, USA) mounting medium. Staining was viewed using an Olympus BX-UCB microscope and QED Imaging software (Meyer Instruments, Houston, TX, USA) modified using Image Pro Plus software (Media Cybernetics, Inc., Bethesda, MD, USA) and quantified using Image Pro Plus software for MAP2 staining and ImageJ software (National Institutes of Health, Bethesda, MD, USA) for synaptophysin staining. Synaptophysin staining was measured as the ratio of the area of immuno-positive staining in the SO relative to the SL. MAP2 staining was quantified as the amount of immuno-positive staining found in the SL. Boundaries of the different regions were defined visually and circled as areas of interest.

Experiment 1: Adult LER and WR males and females

Behavioral Training

The Morris water maze consisted of a white, circular, polypropylene pool measuring 155cm in diameter, 60cm in height. The pool was filled to a depth of 37.5cm.

The platform was made of clear Plexiglas measuring 14cm in diameter. Testing was conducted for 5 days. Latency to reach platform and position of the animal was measured for all animals on all trials. If animals did not reach the platform after 60 seconds, they were guided to the platform and left there for 10 seconds before being removed to a holding cage. All animals were given 8 trials a day with a different start location in the pool for each trial. The sequence of start locations were the same across days. (See Figure 1 for illustration of platform locations during testing days).

Caption Figure 1

Figure 1: Platform locations for adult MWM over the course of 5 days. Red circle indicates platform location for day 1 trials 1-8. Blue platform indicates platform location for days 2-5 trial 1 only. Green circle indicates platform location for days 2-5 trials 2-8.

Lines demarcate the four quadrants of the water maze

Figure 1

Vaginal swabs

Vaginal swabs were taken from adult females following vaginal opening (~p30) to determine phase within the estrous cycle using the lavage technique, as described in Warren & Juraska, 1997 and Warren et al., 1995. Swabs were taken daily until day of euthanasia. Slides of samples were examined using an Olympus BX-UCB microscope using QED Imaging software (Meyer Instruments, Houston, TX, USA). Proestrus was indicated by the majority of cells appearing large, round and nucleated. Estrus was indicated by cells appearing cornified. Finally, diestrus was determined when there appeared to be a variety of cells types along with leukocytes within the samples.

Immunohistochemistry

Adults were euthanized from 2 to 5 days following Morris water maze testing between P46 and 50 (9 LER females, 18 LER males, 11 WR female and 10 WR males) using CO₂ anesthesia and decapitation. Brains were removed and immersion fixed with 4%-paraformaldehyde in 0.1M phosphate-buffered saline (PBS) overnight at 4°C. This solution was replaced with 30% sucrose in 0.1M PBS the following day and brains were stored at 4°C until sectioning.

Experiment 2: Developmental analysis of LER and WR males and females

Pups were tested in the Morris water maze over the developmental time point from p16 to p26 for their acquisition of the platform switching task. Due to the age and hormonal status of the animals, cyclicity of the females in the estrus cycle was not considered.

These same animals were examined for their ability in the MWM task as adults. Behavioural and immunohistochemistry techniques were conducted similarly as previously described in experiment 1.

Behavioral Training

The Morris water maze pool was a white, circular, polypropylene pool measuring 124cm in diameter, 31cm in height and filled with water to a depth of 25cm. The platform was made of clear Plexiglas and measured 11cm in diameter. Rats began behavioral testing at P16. Animals were run in the water maze for 11 days. All animals were given 8 trials a day with a different start location in the pool for each trial. The sequence of start locations was the same across all days. On day 1, the platform was located in the same location for trials 1-8. On day 2, trial 1, the platform was situated in the same location as the previous day. On trial 2, the platform was moved to a new location, where it remained for the following 7 trials. On days 3-10, a similar procedure was used such that the platform was located in the same location for trial 1 as it was for trials 2-8 from the previous day then moved to a new location for trials 2-8 (see Figure 3 for illustration). Latency to reach the platform as well as the position of the animal was recorded for all trials. If the animal did not reach the hidden platform within 60s, they were guided to it. Animals remained on the platform for 10 seconds before being removed and placed into a holding cage for an additional 20 seconds.

Statistical analysis

For adult animals in the MWM, latency to reach the platform (in seconds) and distance traveled during each trial (in meters) were recorded. Data were analyzed initially for within groups differences between animals with or without juvenile experience in the MWM. 2-way mixed design ANOVAs were conducted using strain/gender as a between subjects factor and testing day as a within subjects factor. In order to examine effects of early-life experience in the MWM, 2-way, mixed design ANOVAs were conducted within strain and gender groups using experience as a between subjects factor and testing day as a within subjects factor. Further, in order to examine the effects of estrous cycle on female MWM performance, 3-way mixed design ANOVAs were conducted with strain and gender group and phase in the estrous cycle as between subjects factors and day as a within subjects factor.

For juvenile rats in the MWM, only latency to reach the platform (in seconds) was recorded. Results were analyzed using a mixed-design ANOVA, with the average latency across the 8 trials for each testing day as the within subjects factor and strain and gender group as a between-subjects factor.

The ratio of synaptophysin immuno-positive staining in the SO relative to the SL and the amount of MAP2 immuno-positive particles in the SL were measured for adult animals. An overall 1-way ANOVA was conducted with strain/gender as a between subjects factor. Further, an additional 1-way ANOVA was conducted within individual strain and gender groups with experience as a between subjects factor.

Caption Figure 2

Figure 2: Platform locations for developmental MWM over the course of 11 days (p16 to p26). Red circle indicates platform location on day 1 for trials 1-8. Blue circle indicates platform location for days 2-10 trial 1 only. Green circle indicates platform location for days 2-10 for trial 2-8. Lines demarcate the four quadrants of the water maze

Figure 2

Results

Morris water maze – Overall analysis

Given the unique platform switching MWM used, a specific pattern of behaviour was assumed to indicate appropriate task learning. On the first day, in order to demonstrate that animals were able to learn the task at the same rate, animals, regardless of strain, gender or juvenile experience, should have showed decreasing latencies and shorter path lengths as they progressed from trial 1 to trial 8. On subsequent days, if an animal had learned this task, it should have showed a lower latency and shorter path length on the first trial, demonstrating memory recall of the previous day's platform location. When the platform was moved on trial 2, the animal should have showed high latencies and longer path lengths as they searched for the new platform location. Finally, the latency and path length scores on trials 3 through 8 should have showed decreasing values, demonstrating that the animals had indeed learned the new platform location over the course of the last six trials. In short, latency and path length scores for the first three trials should have showed an “inverted V” shape when represented graphically, with low values on trials 1 and 3 and high values on trial 2. This pattern of behaviour was the focus of this study's analysis.

Morris water maze – Adult rats

MWM switching platform task acquisition was assessed for memory recall over a 24 hour period, learning flexibility and acquisition over the course of five testing days. Latency to reach the platform as well as distance traveled during each trial was measured.

Memory recall was examined through the analysis of scores on trial 1. Lower latencies to reach the platform as well as shorter distances traveled on trial 1 indicated improved memory recall of the platform location from the previous day. Learning flexibility was assessed through the calculation of the latency or distance traveled on trial 3 subtracted from that recorded on trial 2, divided by the sum of the two trials ($\text{trial 2} - \text{trial 3} / \text{trial 2} + \text{trial 3}$). Positive ratios approaching a value of 1 indicated a greater difference between trial 2 and 3, demonstrating quick learning of the new platform location. Small, positive ratios indicated a smaller difference between trial 2 and 3, demonstrating poorer learning of the new platform location. Any negative ratios indicated a greater latency or distance traveled on trial 3 as compared to trial 2, showing poor or no learning of the new platform location from trial 2 to 3 and therefore poor learning flexibility. Acquisition over the course of a single day was assessed through the calculation of the difference of the latency or distance traveled between trials 3 and 8. Smaller difference scores indicated improved task acquisition over the last 6 trials.

Results on trial 1, the ratio of the differences between trial 2 and 3 to the sum of trial 2 and 3, as well as the difference between trial 3 and trial 8, were included in individual analyses. Latency and distance traveled during each trial was plotted across all 8 trials per day across 5 days of training for qualitative analysis of the data. Next, data were analyzed within juvenile experience group, i.e. for either with or without juvenile experience, as a 2-way mixed design ANOVA, with strain and gender as a between subjects factor and day as a within factor. Individual 1-way ANOVAs, using strain and gender as a between subjects factor for each day, were conducted to determine on which days specific group differences existed. Two-way, mixed design ANOVAs were

conducted with experience as a between subjects factor and day as a within subjects factor in order to determine the effect of juvenile experience for each strain and gender group. Individual 1-way ANOVAs were conducted using experience as a between subjects factor for each day of testing in order to determine on which days groups showed significant differences.

Qualitative description of latency and path length

Averages for the latency to reach the platform and the distance traveled during all trials over the course of 5 days of training can be seen in Figures 3 and 4, respectively. Both latency and path length data showed similar trends. LER females are depicted in Fig 3A and 4A. By day 3, LER females with or without juvenile experience in the MWM showed low latencies and short path lengths on trial 1, elevated latencies and path lengths on trial 2, and lower latencies and path lengths from trial 3 through 8. By day 3, LER males (see Fig 3B and 4B) with no juvenile experience showed low latencies and path lengths, on trial 1, high latencies and path lengths on trial 2 and decreasing latencies and path lengths on trials 3 through 8. This “inverted V” behavioural pattern appeared by day 2 in LER males with juvenile experience in the MWM. WR females are depicted in Fig 3C and 4C. WR females with no juvenile experience in the MWM almost show this “inverted V” pattern of behaviour by day 3, although lowered latencies on trial 3 are not seen. This pattern does not persist to days 4 and 5. WR females with juvenile experience in the MWM show this behavioural pattern by day 4, although it is not maintained to day 5. WR males (see Fig 3D and 4D) with no experience in the MWM do not appear to show this behaviour on any day. By day 4, WR males with juvenile experience in the

MWM showed low latencies and path lengths on trial 1, increasing latencies and path lengths on trial 2 and decreasing latencies and path lengths from trial 3 through 8, and this pattern persists to day 5. These results appeared true also for path length data.

Figure 3 Caption

Figure 3: Latency to reach the platform in seconds across all 5 days of testing for adult rats with or without juvenile experience in the MWM. Adults with no juvenile experience (No Exp). Adults with juvenile experience (J Exp) A. LER females B. LER males; C. WR females; D. WR males

Figure 3

Caption Figure 4

Figure 4: Distance traveled in meters across all 5 days of testing for adult rats with or without juvenile experience in the MWM. Adults with no juvenile experience (No Exp). Adults with juvenile experience (J Exp) A. LER females; B. LER males; C. WR females; D. WR males

Figure 4

Due to the similarity between latency and path length data, a correlational analysis was conducted to determine if subsequent analyses should include path length. Thus, the latency and distance traveled on day 2 trial 1 was used for correlational analysis. For animals with no juvenile experience in the water maze, the Pearson correlation coefficient was 0.940, $p < 0.0001$ and for animals with juvenile experience in the MWM, a Pearson correlation coefficient of 0.974, $p < 0.0001$ was obtained. This indicated that both latency to reach the platform and distance traveled during each trial was highly correlated, therefore only latency data were included in all subsequent analyses, since it appeared representative of task performance.

Adult females – Estrous cycle

For adult females, no overall differences were observed between females in different phases of the estrous cycle for any behavioural measure examined. Estrous cycle was not considered in any subsequent analyses.

24 Hour memory recall.

Latency on trial 1 across all days of testing was used to measure 24 hour memory recall and can be seen graphically in Figure 5 for all groups.

Adults with no juvenile experience in the MWM. Latency on trial 1 across all 5 days of testing was examined using a 2-way mixed design ANOVA with strain and gender group as a between subjects factor and day as a within subjects factor. Sphericity was

determined to be violated using Mauchly's test of sphericity, therefore the Huynh-Feldt correction was applied. There was an overall within subject difference, even with the applied correction ($F(3.96, 174.259) = 47.87, p < 0.0001$). No interaction was observed between the day and strain and gender group. The assumption of homogeneity of variance was found to be violated for all days, therefore a more stringent α level of 0.025 was applied. An overall differences between strain and gender groups was observed ($F(3, 44) = 3.881, p = 0.015$). Using the more stringent Bonferroni correction, an overall significant difference was found between LER males and WR females ($p = 0.019$).

Individual 1-way ANOVAs were conducted on each day in order to determine on which days strain and gender groups differed. On day 1, no overall differences were observed between any groups. On day 2, an overall difference was observed between groups ($F(3, 44) = 2.975, p = 0.042$), and specifically, WR females were shown to have significantly higher latencies on trial 1 as compared to LER males ($p = 0.006$) and WR males ($p = 0.047$). No overall differences were observed on day 3, 4 or 5.

Adults with juvenile experience in the MWM. Latency on trial 1 across all 5 days of testing was examined using a 2-way mixed design ANOVA with strain and gender group as a between subjects factor and day as a within subjects factor. Sphericity was determined to be violated using Mauchly's test of sphericity, therefore the Huynh-Feldt correction was applied. Even with the correction, there was an overall within day difference between groups ($F(3.742, 59.869) = 8.265, p < 0.000$). There was no interaction between day and strain and gender group. The assumption of homogeneity of variance was violated on multiple days, therefore a more stringent α level of 0.025 was

applied. With the more stringent α level, there was no overall difference between strain and gender groups for latency on trial 1 ($F(3, 16) = 3.789, p = 0.032$).

Individual 1-way ANOVAs were conducted on each day to determine initial strain and gender group differences for latency on trial 1 for all animals with juvenile experience in the MWM. This can be seen graphically in Fig 5B. No significant differences were observed on day 1 or 2. On day 3, the assumption of homogeneity of variance was violated, therefore a more stringent α level of 0.025 was applied. An almost significant difference between groups was seen ($F(3, 16) = 3.745, p = 0.033$). Upon closer analysis, LER females were found to show almost significantly lower latencies on trial 1 as compared to WR females ($p = 0.038$). As well, LER males were shown to have significantly lower latencies on trial 1 as compared to WR females ($p = 0.009$). Further, WR males were shown to have almost significantly lower latencies on trial 1 as compared to WR females ($p = 0.040$). No overall differences were observed between groups on day 4 or day 5 for latency to reach the platform on trial 1.

Comparing within strain and gender groups. 2-way mixed design ANOVAs were conducted for all strain and gender groups with juvenile experience as a between subjects factor and day as a within subjects factor. Individual 1-way ANOVAs were conducted in order to identify differences within strain and gender groups for the effect of juvenile experience in the MWM on each testing day.

For LER females (see Fig 5C), there was a significant effect of day ($F(4, 48) = 10.311, p < 0.000$) but no interaction between day and juvenile experience. Since the

assumption of homogeneity of variance was violated, a more stringent α level of 0.025 was applied. Even with this correction, there was an overall effect of juvenile experience on latency to reach the platform on trial 1 ($F(1, 12) = 9.778, p = 0.009$). Upon further analysis, it was observed that on day 1, LER females with juvenile experience showed significantly lower latencies to reach the platform trial 1 as compared to LER females with no juvenile experience ($F(1, 12) = 4.512, p = 0.055$). On day 2, the same results were achieved such that LER females with juvenile experience showed significantly lower latencies on trial 1 as compared to LER females without juvenile experience ($F(1, 12) = 4.858, p = 0.048$). No significant differences were found on days 3, 4 or 5.

For LER males (see Fig 5D), there was a significant effect of day ($F(4, 84) = 22.899, p < 0.0001$) but no interaction between day and juvenile experience. Since the assumption of homogeneity of variance was violated, a more stringent α level of 0.025 was applied. Even with this correction, there was an overall effect of juvenile experience on latency to reach the platform on trial 1 ($F(1, 21) = 6.590, p = 0.018$). On day 1, LER males with juvenile experience showed significantly lower latencies to reach the platform on trial 1 as compared to LER males without juvenile experience ($F(1, 21) = 9.732, p = 0.005$). On day 2, LER males with no juvenile experience showed significantly higher latencies to reach the platform on trial 1 as compared to LER males with juvenile experience ($F(1, 21) = 4.842, p = 0.039$). No significant differences were observed on days 3, 4 or 5.

For WR females (see Fig 5E), there was a significant effect of day ($F(4, 56) = 5.961, p < 0.000$) but no interaction between day and juvenile experience. There was no overall effect of juvenile experience on the latency to reach the platform on trial 1 for

WR females. Individual 1-way analyses were conducted on each day, and it was observed that on day 1 WR females with juvenile experience had significantly lower latencies to reach the platform as compared to WR females with no juvenile experience ($F(1, 14) = 4.923, p = 0.019$). On day 2, WR females with juvenile experience were shown to have significantly lower latencies on trial 1 as compared to WR females with no experience ($F(1, 14) = 6.108, p = 0.027$). No significant differences were observed on days 3, 4 or 5.

For WR males (see Fig 5F), there was a significant effect of day ($F(4, 52) = 5.829, p = 0.001$) but no interaction between day and juvenile experience. There was no overall effect of juvenile experience on the latency to reach the platform on trial 1 for WR males. On day 1, WR males with no juvenile experience were shown to have significantly lower latencies on trial 1 as compared to WR males with no juvenile experience ($F(1, 13) = 4.799, p = 0.047$). No significant differences were observed on days 2, 3, 4 or 5.

Figure 5 Caption

Figure 5: Latency to reach the platform in seconds on trial 1 across all 5 days of testing for adult rats with or without juvenile experience. Adults with no juvenile experience (No Exp). Adults with juvenile experience (J Exp) A. Adult rats with no juvenile experience. B. Adult rats with juvenile experience. C. LER females with and without juvenile experience. D. LER males with and without juvenile experience. E. WR female with and without juvenile experience. F. WR males with and without juvenile experience. *

Indicates a significant difference at the $p = 0.025$ level.

Figure 5

Learning flexibility

The ratio of the difference of the latency on trial 2 and trial 3 to the sum of the latencies on trial 2 and trial 3 across all 5 days of testing can be seen in Figure 6 for all groups of animals.

Adults with no juvenile experience in the MWM. There was no overall effect of day on this ratio. As well, there was no interaction between the day and the gender and strain group. No overall differences were observed between groups for this ratio.

Individual 1-way ANOVAs were conducted on each day in order to determine individual differences between groups (see Fig 6A). No overall differences were observed on day 1 and 2. On day 3, overall differences were observed between groups ($F(3, 44) = 4.301, p = 0.010$). Both LER females and LER males were found to have a higher ratio as compared to WR females (LER females *cf* WR females, $p = 0.002$; LER males *cf* WR females, $p = 0.007$). No overall differences were observed between groups on day 4 or day 5.

Adults with juvenile experience in the MWM. There was no overall effect of day, strain and gender group, nor was there any interaction between day and gender group for the ratio of the difference of the latency on trial 2 and trial 3 to the sum of the latencies on trial 2 and trial 3.

Individual 1-way ANOVAs were conducted on each day in order to determine differences in this ratio between animals with juvenile experience in the MWM (see Fig 6B). No overall differences were observed between groups on day 1, 2, 3, 4 or 5.

Comparing within strain and gender groups. Mixed design, 2-way ANOVAs were conducted for all strain and gender groups with juvenile experience as a between subjects factor and day as a within. Individual 1-way ANOVAs were conducted to identify differences within strain and gender groups for the effect of juvenile experience in the MWM for each individual day.

For LER females (see Fig 6C), there was no significant effect of day, juvenile experience or an interaction between day and juvenile experience for the trial 2/3 ratio. There were no significant differences between the ratio on days 1 and 2. On day 3, LER females with juvenile experience showed significantly lower ratios than LER females with no juvenile experience ($F(1, 12) = 6.284, p = 0.028$). No differences were observed on days 4 and 5.

For LER males (see Fig 6D), there was no significant effect of day, juvenile experience or an interaction between day and juvenile experience for the day 2/3 ratio. When individual days were examined, there were no significant differences between LER males with or without juvenile experience in the MWM on all days.

For WR females (see Fig 6E), there was no significant effect of day, juvenile experience or an interaction between day and juvenile experience for the difference ratio. On day 1, it was observed that WR females with juvenile experience in the MWM showed significantly smaller ratios as compared to WR females with no juvenile experience ($F(1, 14) = 4.923, p = 0.044$). No significant differences were observed on day 2, 3, 4 or 5.

For WR males (see Fig 6F), there was no significant effect of day, juvenile experience, or an interaction between day and juvenile experience for the day 2/3 ratio.

Upon examination of individual analyses for each day, no differences were observed on any day.

Figure 6 Caption

Figure 6: Ratio of the difference of the latencies on trial 2 and trial 3 to the sum of the latencies on trial 2 and trial 3 across all 5 days of testing for adult rats with or without juvenile experience in the MWM. Adults with no juvenile experience (No Exp). Adults with juvenile experience (J Exp). A. Adults with no juvenile experience. B. Adults with juvenile experience. C. LER females with and without juvenile experience. D. LER males with and without juvenile experience. E. WR female with and without juvenile experience. F. WR males with and without juvenile experience. * Indicates a significant difference at the $p = 0.025$ level.

Figure 6

Within-day Acquisition

A plot of the difference scores for the latencies observed on trial 3 and trial 8 can be seen in Figure 7 for all animals.

Adults with no juvenile experience in the MWM. A 2-way mixed design ANOVA was run using strain and gender group as a between subjects factor and day as a within factor. The assumption of sphericity was violated using Mauchly's test of sphericity, therefore the Huynh-Feldt correction was applied. With the correction applied, there was no overall within day difference nor was there any interaction between day and strain and gender group. There was no overall difference between strain and gender groups.

Individual 1-way ANOVAs were conducted for each day, and can be seen in Fig 7A for animals with no juvenile experience in the MWM. On day 1 and 2, no overall differences were observed between groups. On day 3, an overall difference was observed between groups for the within-day acquisition ($F(3, 44) = 7.364, p < 0.000$). WR females showed a much larger difference between the latency score on trial 3 and trial 8 as compared to all other groups, indicating superior acquisition of the WR females (LER females *cf* WR females, $p < 0.000$; LER males *cf* WR females, $p = 0.002$; WR males *cf* WR females, $p < 0.000$). No significant differences were observed on day 4 or 5.

Adults with juvenile experience in the MWM. There was no overall effect of day nor was there any interaction between day and strain and gender group. There was no

overall effect of strain and gender group for the difference between the latencies on trial 3 and trial 8.

Individual 1-way ANOVAs were conducted in order to determine deviations of groups from one another in terms of the difference between the latency on trial 3 and trial 8 (see Fig 7B). No overall differences were observed on any day.

Comparing within strain and gender groups. Mixed design, 2-way ANOVAs were conducted for all strain and gender groups with juvenile experience as a between subjects factor and day as a within subjects factor.

For LER females (see Fig 7C), there was no significant effect of day, juvenile experience or an interaction between day and juvenile experience for the difference score. No significant differences were observed between LER females with or without juvenile experience on days 1 or 2. On day 3, LER females with juvenile experience showed significantly larger difference scores between trials 3 and 8 latencies, demonstrating superior acquisition as compared to LER females with no juvenile experience in the MWM ($F(1, 12) = 5.532, p = 0.037$). No specific differences were observed within LER females for days 4 or 5.

For LER males (see Fig 7D), there was no significant effect of day, juvenile experience or an interaction between day and juvenile experience for the difference score. There were no significant differences between LER males with or without juvenile experience on any day of testing.

For WR females (see Fig 7E), there was no significant effect of day or juvenile experience, but the interaction between day and juvenile experience approached

significance ($F(4, 56) = 2.385, p = 0.062$). On day 1, WR females with no juvenile experience showed significantly larger difference scores demonstrating better acquisition as compared to WR females with juvenile experience ($F(1, 14) = 4.618, p = 0.050$). No significant differences were observed on day 2. On day 3, WR females with no juvenile experience once again showed larger difference scores compared to WR females with juvenile experience ($F(1, 14) = 5.077, p = 0.041$). No differences were observed on days 4 or 5.

For WR males (see Fig 7F), there was neither a significant effect of day, juvenile experience nor an interaction between day and juvenile experience for the difference score. No individual differences were observed on any day.

Figure 7 Caption

Figure 7: Difference scores of trial 3 and trial 8 latencies in seconds across all 5 days of testing for adult rats with or without juvenile experience in the MWM. Adults with no juvenile experience (No Exp). Adults with juvenile experience (J Exp) A. Adults with no juvenile experience. B. Adults with juvenile experience. C. LER females with and without juvenile experience. D. LER males with and without juvenile experience. E. WR females with and without juvenile experience. F. WR males with and without juvenile experience. * Indicates a significant difference at the $p = 0.025$ level.

Figure 7

Developmental MWM

Only latency to reach the platform was recorded during the developmental MWM testing. 2-way mixed design ANOVAs were conducted with strain and gender group as a between subjects factor and the average latency score across all 8 trials in one testing day as the within. Overall, there was a significant effect of day, even when the Huynh-Feldt correction was applied due to a violation of sphericity ($F(8.801, 176.015) = 103.262, p < 0.000$). There was also a significant interaction between day and strain and gender group with the Huynh-Feldt correction applied ($F(26.402, 176.015) = 3.946, p < 0.000$). With an adjusted α level of 0.025 due to a violation of homogeneity of variance, there was a significant overall effect of strain and gender group ($F(3, 20) = 17.415, p < 0.000$). Specific comparisons between groups were conducted revealing that LER females showed significantly lower latencies than WR females ($p < 0.0001$) and WR males ($p < 0.0001$). LER males also showed significantly lower latencies than WR females ($p = 0.001$) and WR males ($p < 0.0001$).

Individual 1-way ANOVAs were conducted from p18 (day 3 of testing) onwards to determine when individual group differences arose. On p18 and p19, no significant differences between groups were observed. Significant overall differences were observed on p20 ($F(3, 20) = 9.470, p < 0.000$) with LER females and LER males showing significantly lower latencies than both WR females and WR males (LER females *cf* WR females, $p = 0.006$; LER females *cf* WR males, $p = 0.011$; LER males *cf* WR females, $p = 0.005$; LER males *cf* WR males, $p = 0.009$). These overall and specific differences were observed on p21 (overall: $F(3, 20) = 10.617, p < 0.000$; specific: LER females *cf* WR

females, $p = 0.003$; LER females *cf* WR males, $p < 0.000$; LER males *cf* WR females, $p = 0.008$; LER males *cf* WR males, $p < 0.000$), p22 (overall: $F(3, 20) = 12.461$, $p < 0.000$; specific: LER females *cf* WR females, $p = 0.001$; LER females *cf* WR males, $p = 0.003$; LER males *cf* WR females, $p = 0.002$; LER males *cf* WR males, $p = 0.004$), p23 (overall: $F(3, 20) = 19.642$, $p < 0.000$); specific: LER females *cf* WR females, $p < 0.000$; LER females *cf* WR males, $p = 0.002$; LER males *cf* WR females, $p < 0.000$; LER males *cf* WR males, $p = 0.001$), p24 (overall: $F(3, 20) = 11.087$, $p < 0.000$; LER females *cf* WR females, $p = 0.002$; LER females *cf* WR males, $p = 0.015$; LER males *cf* WR females, $p = 0.001$; LER males *cf* WR males, $p = 0.006$) and p25 (overall: $F(3, 20) = 6.814$, $p = 0.002$; specific: LER females *cf* WR females, $p = 0.020$; LER females *cf* WR males, $p = 0.045$; LER males *cf* WR females, $p = 0.017$; LER males *cf* WR males, $p = 0.038$). On p26, there was an overall difference between groups ($F(3, 20) = 8.278$, $p = 0.001$), but LER females and males showed significantly lower latencies than only the WR males (LER females *cf* WR males, $p = 0.004$; LER males *cf* WR males, $p = 0.005$), although the difference between the LER females and LER males as compared to the WR females approached significance (LER females *cf* WR females, $p = 0.053$; LER males *cf* WR females, $p = 0.063$).

Figure 8 Caption

Figure 8: Latency to reach the platform averaged across 8 trials for all 11 days of testing (p16 to p26). * Indicates a significant difference at 0.025 α level between the LER females and the WR females. ** Indicates a significant difference at 0.025 α level between the LER females and the WR males. # Indicates a significant difference at 0.025 α level between the LER males and the WR males. ## Indicates a significant difference at 0.025 α level between the LER males and the WR females.

Figure 8

Immunohistochemistry

An overall view of the rat brain can be seen in Figure 9, with the particular areas of interest highlighted for the individual immunohistochemical analyses.

Individual one-way ANOVAs were conducted for the ratio of immuno-positive synaptophysin staining in the SO relative to the SL and the amount of immuno-positive MAP2 staining in the SL, with strain and gender as a between subjects factor. If the assumption of homogeneity of variance was violated, a more stringent α level of 0.025 was used. A ratio of synaptophysin staining in the SO relative to the SL approaching a value of 1 signified increased axonal innervation (by the MFs) of the SO relative to the SL. The higher the amount of immuno-positive staining of MAP2 in the SL indicated potential increased dendritic branching in this area of the hippocampus.

Figure 9 Caption

Figure 9: Sample images for immunohistochemistry staining. A. Cresyl violet stain at 4X magnification. Red box demarcates the CA3 area of interest. B. Immunofluorescence staining of the CA3 area of interest at 20X magnification. DAPI stain (blue), synaptophysin (red), MAP2 (green). C. DAB staining for synaptophysin of the CA3 area of interest at 20X magnification. Second picture shows circled areas of interest. Red line demarcates the SL region of the CA3 and the green line demarcates the SO. D. DAB staining for MAP2 of the CA3 region at 20X magnification. Blue line demarcates the SL area of interest.

Figure 9

Synaptophysin staining in the SO relative to the SL

Adults with no juvenile experience. Individual 1-way ANOVAs were conducted in order to compare the ratio of synaptophysin immuno-positive staining in the SO relative to the SL for animals with no juvenile experience in the MWM. A graphical representation of this can be seen in Figure 10A. No overall differences were observed between groups.

Adults with juvenile experience. Individual 1-way ANOVAs were conducted in order to compare the ratio of immuno-positive synaptophysin staining in the SO relative to the SL for animals with juvenile experience in the MWM. A graphical representation of this can be seen in Figure 10B. No overall differences were observed between groups.

Comparing within strain and gender groups Individual 2-way ANOVAs were conducted in order to determine differences between animals with or without juvenile experience in the MWM within the same strain and gender group. Graphical representations of these findings can be seen in Fig 10C, D, E and F for LER females, LER males, WR females and WR males, respectively. No differences were observed between LER females or males regardless of juvenile experience. Overall differences were found between WR females with or with juvenile experience ($F(1, 17) = 21.562, p < 0.000$) such that WR females with juvenile experience had significantly greater ratios of immuno-positive synaptophysin staining in the SO relative to the SL as compared to WR

females with no juvenile experience. WR males with juvenile experience were found to have significantly smaller ratios of immuno-positive synaptophysin staining in the SO relative to the SL ($F(1, 15) = 13.413, p = 0.002$).

Figure 10 Caption

Figure 10: Ratio of synaptophysin immuno-positive staining in the stratum oriens (SO) relative to the stratum lucidum (SL) for adult rats with or without juvenile experience in the MWM. Adults with no juvenile experience (No Exp). Adults with juvenile experience (J Exp). A. Adults with no juvenile experience. B. Adults with juvenile experience. C. LER females with and without juvenile experience. D. LER males with and without juvenile experience. E. WR females with and without juvenile experience. F. WR males with and without juvenile experience. * Indicates a significant difference at the $p = 0.025$ level.

Figure 10

MAP2 staining in the CA3

Adults with no juvenile experience. Individual 1-way ANOVAs were conducted in order to compare the amount of MAP2-immuno positive staining in the CA3 region of the hippocampus in animals with no juvenile experience. A graphical representation of this can be found in Figure 11A. No overall group differences were observed.

Adults with juvenile experience. Individual 1-way ANOVAs were conducted in order to compare the amount of MAP2-immuno positive staining in the CA3 region of the hippocampus in animals with juvenile experience. A graphical representation of this can be seen in Figure 11B. Overall differences were observed between groups ($F(3, 29) = 5.991, p = 0.003$). LER males were found to have significantly higher levels of MAP2 immuno-positive staining in the SL as compared to all other groups, as determined by LSD post hoc tests (LER males *cf* LER females, $p < 0.000$; LER males *cf* WR females, $p = 0.037$; LER males *cf* WR males, $p = 0.016$).

Comparing within strain and gender groups. Individual 1-way ANOVAs were conducted in order to determine the effect of juvenile experience on MAP2 immuno-positive staining in the SL region of the CA3. These can be seen graphically in Fig 11C, D E and F for LER females, LER males, WR females and WR males respectively. LER females with no experience were found to have significantly higher levels of MAP2 staining in the SL ($F(1, 15) = 9.362, p = 0.008$). LER males with juvenile experience were found to have significantly higher levels of MAP2 staining in the SL as compared to

LER males with no experience ($F(1, 14) = 15.782, p = 0.001$). No differences were observed for WR females and WR males.

Figure 11 Caption

Figure 11: Microtubule-associated protein 2 immuno-positive staining in the stratum lucidum in adult rats with or without juvenile experience in the MWM. Adults with no juvenile experience (No Exp). Adults with juvenile experience (J Exp). A. Adults with no juvenile experience. B. Adults with juvenile experience. C. LER females with and without juvenile experience. D. LER males with and without juvenile experience. E. WR females with and without juvenile experience. F. WR males with and without juvenile experience. * Indicates a significant difference at the $p = 0.025$ level.

Figure 11

Discussion

Results Summary

Developmental MWM. Strain differences were observed for animals for MWM training during development. LER and WR strains, regardless of gender, showed similar MWM behaviour from p16 to p19. From p20 onwards, LER demonstrated superior task acquisition as compared to WR.

No juvenile MWM experience. By day 3, LER males and females demonstrated MWM task acquisition, whereas WR males and females, never showed behavioral patterns indicative of learning the task.

Quantitatively, LER males innately showed improved scores as compared to WR females and males when 24-hour memory recall was examined. These innate differences were observed only on day 2 of training. On day 3, LER males and females showed increased learning flexibility as compared to WR males. WR females showed superior daily learning acquisition as compared to all other groups on day 3.

Juvenile MWM experience. LER and WR males with juvenile MWM experience demonstrated acquisition one day earlier compared to their counterparts with no juvenile experience.. LER and WR females did not show any benefit given juvenile MWM experience. These initial results demonstrate that males may benefit the most from early-life MWM experience.

Quantitatively, LER males with juvenile experience showed an advantage over WR females with juvenile experience when 24-hour memory recall was measured on day

3. All groups with juvenile experience, save for WR males, showed improved 24-hour memory recall on day 2. Differences observed between the LER strain and WR males seen in animals with no juvenile MWM experience were no longer apparent once animals were exposed to juvenile experience, with the WR males with juvenile pre-training demonstrating more “LER-like” behaviour. LER females were the only group to show significantly decreased learning flexibility on day 3 as a result of early-life MWM experience. Observed differences between WR males and all other groups with no juvenile MWM experience were not seen in animals with juvenile MWM experience in terms of learning acquisition on any day.

Surprisingly, LER females showed deficits as a result of juvenile MWM experience in terms of acquisition on day 3. LER females also demonstrated decreased dendritic branching due to juvenile MWM experience.

WR females showed increased axonal arborizations in SO relative to SL as a result of juvenile experience, whereas WR males showed the opposite effect. LER males with juvenile experience in the MWM showed increased dendritic branching as compared to all other groups with juvenile MWM experience.

Developmental MWM differences

From p16 to p19, no overall difference was observed between any of the groups when measuring time required to locate the hidden platform. At p20, the learning behaviour of the two strains split. LER males and females showed significantly lower latencies to reach the platform as compared to WR males and females, an effect which lasted until p26. These results parallel earlier studies of LER and WR males MF

development. LER and WR males showed the same pattern of innervation of the CA3 region at p21, with MFs extending to both the SL and distal SO of CA3 (Holahan et al., 2007). By p24, the MFs of the WR males had retracted from SO to innervate solely the SL (Holahan et al., 2007). Combined with the results achieved here, it appears that even though morphologically, LER and WR show similar MF innervation patterns of the CA3 region from p20-p24, WR are not able to show the same level of spatial learning as LER during development. This demonstrates that the WR SO projection may not provide for the same activity-dependent processes that result in its stabilization or aid in spatial learning. Specifically, LER as compared to WR show much lower latencies over the course of development, indicating improved maze performance. This improvement in behaviour may be due to increased innervation of the SO region of the CA3 by the MFs found in the LER. This pattern of innervation is correlated not only with enhanced synaptic efficacy (Heimrich et al., 1989) but is also well known for its role in complex spatial learning (Crusio et al., 1987; Crusio & Schwegler, 1987; Jacobs et al., 1990; Schwegler et al., 1990; Schöpke et al., 1991; Schwegler & Crusio, 1995; Cantallops & Routtenberg, 1999; Roskoden et al., 2005). As well, there was no gender difference observed in either strain. This may be explained by the fact that neither males nor females have entered puberty at this age (Korenbrod et al., 1977; Warren & Juraska, 1997).

This MWM switching task may be a useful tool for examining the development of different structures in the brain that relate to spatial and working memory. Since this task can assess an animal for three different cognitive components including 24-hour memory recall, working memory and daily acquisition, it may be useful for examining not only

the development of the hippocampus and the resultant behavioural outputs, but may help examine when other neuronal structures demonstrate functional output during development in both genders and different rat strains. For example, the medial prefrontal cortex is well known for its role in behavioural flexibility (see Kolb, 1984 for review). Future studies could examine developmental behaviour in order to examine the functional development of the prefrontal cortex for its role in this switching platform task, with particular emphasis on the behavioural flexibility component.

Future research should attempt to examine how to make the WR more “LER-like” both in terms of hippocampal development and resultant spatial learning ability. Methods to this end could include both behavioural and pharmacological means. Earlier studies have demonstrated that administration of the NMDA receptor antagonist 3-(2-carboxypiperazin-4-yl) propyl-1-phosphonic acid (CPP) from p17 to p20 in WR males resulted in increased MFTF staining in the SO at p40 (Holahan et al., 2007). Administration of CPP to LER males during this same developmental time point resulted in decreased staining of the SO at p40 (Holahan et al., 2007). Therefore, the structural remodeling of this area in both strains is dependent on NMDA receptor (Holahan et al., 2007). Additional research might focus on the effect of CPP administration to female LER and WR rats or the effects of NMDA receptor agonists during this early developmental time point. As well, the effect of other receptor antagonists or agonists found in the CA3 region during this developmental time point could also help elucidate the developmental progression of this area. A candidate could be cannabinoid receptors, due to their widespread expression in the mammalian hippocampus (Mailleux & Vanderhaeghen, 1992). Other potential treatments for enhancing the spatial learning behaviour of WR might include the use of hormones. For

example, when estrogen is at its highest during proestrous, synapse number in the female brain also peaks (Warren & Juraska, 1997). Therefore, if WR males and females are given estradiol during this same critical developmental period (from p17 to p20), they may show improvements in the MWM platform switching task combined with hippocampal structural remodeling. In addition, administration of testosterone, a hormone which has been shown to increase the thickness of the GCL of the DG and improve female MWM performance (Roof & Havens, 1992), during this critical period may result in similar morphological changes in the CA3 region.

Effect of juvenile experience on brain and behaviour

LER males followed by WR males appeared to show the most benefit from juvenile MWM experience in terms of spatial learning. Overall, females of either strain did not appear to show the same levels of improvements in their learning ability due to juvenile MWM experience as LER males. It should be pointed out that all groups showed a benefit of early life experience when measuring latency to reach the platform on day 1. This difference may simply be an effect of improved swimming ability or task familiarity, which resulted in lowered levels of stress elicited by MWM testing. For animals with no juvenile MWM experience, poor performance on the first day of training could have reflected elevated stress levels. Increased levels of stress hormones, specifically cortisol, are negatively correlated with MWM learning (Roskoden et al., 2005). Juvenile MWM experience may have acted as a form of early-life handling and daily maternal separation, two factors linked with improved MWM learning in adults (Issa et al., 1990; Zaharia et al., 1996). Early-life handling is associated with a lowered

stress response later in life, explaining why animals with early-life MWM experience may have experienced less stress when re-exposed to the MWM as adults (Meaney & Aitken, 1985). Performance on day 1 may be influenced by elevated stress levels and juvenile MWM experience may act to decrease stress to facilitate task performance. Future studies should examine the effect of blocking corticosterone receptors in the hippocampus in both of these strains to see how this may affect MWM behaviour on day 1 with and without juvenile MWM experience.

On day 2, all groups showed lowered latencies on trial 1 due to juvenile MWM experience, save for the WR males. Differences on trial 1 of day 2 are indicative of memory recall for the previous platform's location, since all animals were familiar with the task. Thus, it appears that only the WR males were unable to benefit from early-life MWM experience in terms of improvements in memory recall on day 2. This is consistent with earlier literature showing that even though pups trained and tested in a radial arm maze from p16 to p20 could not show learning behaviour, when tested from p22 to p25, they performed significantly better when compared to age-matched, untrained controls (Carman & Mactutus, 2001).

In terms of learning flexibility, no groups showed any benefit due to early-life MWM experience. These results may reflect a weakness of the learning flexibility metric. If low latency scores are observed on both trial 2 and 3, the resultant calculated ratio will be very small. Therefore, if animals quickly find the platform on trial 2, they may demonstrate lower measures of learning flexibility. Other measures of learning flexibility, including the examination of percent time spent in the quadrant where the

previous day's platform was located as well as measures of thigmotaxis might help to improve the reliability of this measure.

Juvenile MWM experience offered very little benefit to any group in terms of daily task acquisition. It is possible that juvenile MWM experience does not result in drastic changes in either swimming ability and/or spatial navigation skills and only offer improvements on 24-hour memory recall. These results may also reflect a weakness in the measure of daily acquisition. If an animal learns quickly on trial 2 where the new platform location is, they will take less time on trial 3 to find it, and therefore likely take less time on trial 8, resulting in a low difference score. A more appropriate measure would be to examine the slope of the line for latencies measured on trials 2 through 8 for latencies. Steep slopes would demonstrate quick task learning whereas more gradual slopes would indicate relatively slow acquisition.

Juvenile experience in the MWM resulted in lowered levels of dendritic arborizations in the SL for LER females and increased dendritic arborization in the SL for LER males. The results observed in females may reflect differences in the cyclicity of the tested females such that more females with juvenile experience may have been sacrificed during estrus when synapse numbers were at their lowest (Warren & Juraska, 1997); unfortunately, the estrous state on the day of euthanasia was not evaluated. In LER males, increased dendritic branching in SL may indicate increased innervation of the CA3 pyramidal cells by the axons of the DG GCs. Because no differences were observed in the axon terminals of male LER with or without juvenile MWM experience, it suggests that early-life MWM learning resulted solely in dendritic changes. It may be that these animals at an early age showed increased axonal remodeling following MWM

pre-training and changes in dendritic morphology followed thereafter and were reflected in the results. Future research might examine hippocampal morphology in animals with juvenile experience not only following MWM pre-training but several weeks thereafter in order to examine the short- and long-term effects of MWM pre-training.

Within the WR strain, juvenile experience resulted in increased axonal arborization in the SO relative to the SL in females and decreased axonal arborization in the SO relative to the SL in the males. The increased axonal arborizations in the WR females is consistent with earlier research that observed increased staining in the SO in WR due to MWM experience, however, these results were achieved using WR males. It appears that the results achieved for the WR males runs contrary to these findings (Holahan et al., 2006). The differences in these results may have reflected a weakness in this study's measuring technique. Here, the amount of axonal innervation in the SO and SL was measured using synaptophysin immunopositive staining. Synaptophysin is a marker for presynaptic terminals and showed no specificity to the MFs whereas, Holahan et al., 2006, found elevated MF innervation of the SO due to MWM experience using Timm's zinc stain, which is specific to MFs in the CA3. Future research examining the MF innervation pattern in this area should use markers for elevated levels of zinc, a characteristic of the MFs. Differences observed between these results and results presented in Holahan et al., 2006, could also be due to differences in the time of euthanasia following MWM training. In the present study, animals were sacrificed 48 hours after MWM training, whereas Holahan et al., 2006 sacrificed their animals 7 days following training. The results achieved here may simply demonstrate morphological changes in the hippocampus 48 hours after MWM training.

Innate gender differences

Within the LER strain, both males and females with no juvenile experience displayed similar patterns of behaviour. Both LER males and females showed relatively quick task learning, demonstrating similarities in 24-hour memory recall, heightened sensitivity to the new platform location by demonstrating high latencies on trial 2 and low latencies on trial 3, and comparable within-session learning. LER males and females also shared similar scores for the number of axon terminals and the amount of dendritic branching in the CA3 region.

These results are consistent with the literature, in that, if the phase of the estrous cycle is not taken into consideration, few behavioural differences are observed between LER males and females (Bucci et al., 1995; Warren & Juraska, 1995). However, gender differences were not observed in terms of axonal morphology where they were expected such that males have been observed to show larger axonal terminal fields in the CA3 region (Hajszan et al., 2007). Since the number of synapses in the CA3 region fluctuates over the course of the estrous cycle (Warren & Juraska, 1997), it may be that these results reflected the plastic nature of the female rat brain.

Gender differences were more apparent in the WR strain given no juvenile MWM experience. Both male and female WR were unable to show behavioural patterns demonstrative of task acquisition on any training day. However, WR females showed deficits in terms of latency to reach the platform on day 2 as compared to their male counterparts. This suggests that WR males showed improved memory recall over a 24-

hour period compared to WR females. These gender differences were not observed on any other day.

A caveat to this study is that large numbers were not obtained for the different phases of the estrous cycle. These small numbers may in fact have determined the null results achieved. Additional research should examine differences between females of both strains for their MWM performance at all phases of the estrous cycle.

One gender difference observed in both strains, but more seen more drastically in the WR strain, was that males benefited from early-life water maze experience. Specifically in the WR strain, males with juvenile MWM experience showed the learning pattern on days 4 and 5 while WR females did not benefit from early water maze experience. Earlier research demonstrated that following traumatic brain injury, adult male Sprague-Dawley rats (an albino strain) showed enhanced spatial memory performance following 2 weeks of exposure to an enriched environment, whereas females showed no such benefit (Wagner et al., 2002). As well, male Sprague-Dawley rats exposed to enriched environments postnatally showed improvements in a social discrimination task, whereas females were unable to show any benefit (Peña et al., 2009). These results further support the notion of females demonstrating no benefit to enriched environments, such as juvenile MWM experience, on later learning and memory behaviour.

Innate strain differences

Some strain differences were observed between animals with no juvenile MWM experience. LER were able to show appropriate maze learning by day 3, whereas WR

never demonstrated proper learning at all. No differences were observed in terms of dendritic and axonal morphology in the CA3 region. These results were not consistent with the literature where morphological differences have been observed between these two strains such that LER males have been reported to show higher levels of axonal innervation of SO compared to WR males (Holahan et al., 2006; 2007). No differences observed here may be indicative of a weakness of the method for measuring axonal and dendritic morphology. Examining similarly stained sections using non-biased stereology or dendritic tracings might give a more appropriate and precise method for quantification in these areas.

Differences between the strains also emerged when animals were exposed to juvenile MWM experience. LER males and females showed appropriate learning by days 2 and 3 respectively, whereas WR males and females showed this pattern of behaviour by day 4. LER males also demonstrated higher levels of dendritic arborizations in the CA3 region as compared to both WR males and females. Therefore, it appears that handling and juvenile MWM experience may have caused these two strains to react differently in terms of their structural remodeling of the hippocampus. It could be that juvenile experience accentuated the differences between these two strains. Another possibility is that, in this case, “the rich get richer” and LER males, who innately showed increased number of synaptic terminals in the CA3 region, benefited more from early life experience in terms of dendritic morphology of this region than either males or females of the WR strain.

Further research should continue to highlight differences between these two widely used strains for differences in maternal rearing. WR mothers may be less or even more

attentive to their pups which could have a direct action on juvenile and adult spatial learning ability. As well, the stress and anxiety levels between the LER and WR strains would be an important avenue of exploration. Perhaps WR animals do not show the same acquisition rates due to increased stress reactivity and anxiety elicited by this testing procedure. Of important note is the reduced visual acuity of the WR due to albinism (Prusky et al., 2002). Although both strains perform similarly in a visible MWM platform task, it is conceivable that WR are simply unable to discern the supposively salient external cues during MWM spatial learning. These known deficits in visual acuity support the notion of eliminating the use of WR from studies requiring some level of visual acuity, including spatial memory research.

Conclusions

In conclusion, this study attempted to elucidate both the innate and acquired differences between two widely used rat strains in terms of hippocampal morphology and spatial learning ability. These results may help clarify the effect of juvenile environmental enrichment in humans. Thus, some children may innately benefit more than other children when exposed to enriched environments at an early age. Further, both males and females may benefit differently from early-life experiences. The testing paradigm used may also prove useful for the examination of multiple memory systems, which examines the interplay between multiple structures involved in memory consolidation, including the hippocampus. Results here demonstrated that those animals with increased dendritic and/or axonal arborization in the CA3 region of the hippocampus perform the best on the water maze task. The MWM task used in the present thesis is an

improvement over the standard fixed platform version as it is able to take multiple measures in the same animal repeatedly. Finally, the present work highlights the importance of including males and females in learning and memory research, regardless of the weight of work associated with this addition.

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Appendix A

CARLETON UNIVERSITY ANIMAL CARE COMMITTEE

APPLICATION FOR USE OF LIVE ANIMALS IN RESEARCH & TEACHING
- ANIMAL USE PROTOCOL -

PROTOCOL I.D.# P06-12

PART I**1. TITLE OF PROJECT**

Learning-induced presynaptic morphological plasticity in hippocampal mossy fibers

2. PRINCIPAL INVESTIGATOR

Name: Matthew R. Holahan

Position: Assistant Professor

Department & Phone #: Psychology/ Institute of Neuroscience; ext 1543

3. ANIMALS (anticipated requirements)

Species	Strain	Age/Weight	Sex	Total No. for project
1. Rat (<i>Rattus norvegicus</i>)	Wistar	21days	Male	300
2. Rat (<i>Rattus norvegicus</i>)	Wistar	90 days	Male	870
3. Rat (<i>Rattus norvegicus</i>)	Wistar	1.5 year	Male	430

4. CLASS OF ANIMAL USE & MAX. CATEGORY OF INVASIVENESS (according to CCAC)

Maximum Duration of Survival:	Max. Invasiveness (A-E)
Chronic ☒ Acute ☐	C

5. PROJECT PERIOD ANTICIPATED

From: Dec 2006

To: Dec 2009

6. DECLARATION

All animal manipulations described in this protocol will be carried out by trained and competent personnel. All animals in this project will be cared for and used in accordance with the principles of the Canadian Council on Animal Care stated in the "Guide to the Care and Use of Experimental Animals."

It is understood that the protocol number assigned to this submission will be used when ordering animals, and that these animals will be used only for the project described.

Principal Investigator

Date

Appendix B Caption

Schematic representation of the hippocampus. Red line represents a GC axon (MF) extending from the DG to innervate a pyramidal cell dendrite (shown in green) in the SL

Appendix B