Hippocampal CA1 cytoarchitecture and behaviour after combined neonatal cholinergic lesion and environmental enrichment in rats

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Abstract

The effects of neonatal cholinergic lesion and environmental enrichment on rat behaviour and hippocampal morphology were determined. Rats were injected with the immunotoxin 192 IgG- saporin (192S) on postnatal day 7 to selectively lesion forebrain cholinergic neurons and after weaning, were placed in enriched or standard housing for 42 days. Enriched animals, regardless of whether or not they had received 192S, showed significantly enhanced performance on the working memory version of the Morris water maze. This experiment indicated that neonatal 192S lesioning of forebrain cholinergic terminals does not affect spatial learning/memory, a result that is consistent with previous research. As expected, enrichment enhanced spatial learning/memory in control rats and interestingly, also did so for the lesioned rats. The neonatal cholinergic lesion significantly reduced neurogenesis in the dentate gyrus, a result similar to that seen after such a lesion in adulthood. Enrichment did not have a significant effect on neurogenesis although there was an apparent trend towards increased neurogenesis in the non-lesioned animals. The mean number of newborn neurons in the enriched and impoverished lesion groups were identical and both means significantly differed from the enriched non-lesioned mean, suggesting that the lesioned group was resistant to the effects of enrichment. Enrichment had several effects on hippocampal cytoarchitecture but these were selective changes for certain branch orders rather than global alterations. The most interesting consequence of enrichment, in terms of its implication for synapse density and information processing capability, is the increased spine density and spine number observed on some branches of the apical tree. This was evident only in the non-lesioned

rats. Thus, neonatal cholinergic lesion rendered the rats unresponsive to the effects of enrichment on cell morphology and hippocampal plasticity but not spatial memory.

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192S	192 IgG saporin
Ach	acetylcholine
AchE	acetylcholinesterase
ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
BrdU	bromodeoxyuridine
CHAT	choline acetyltransferase
DCX	doublecortin
Dil	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
GDNF	glial-cell derived neurotrophic factor
E	enrichment condition
I	impoverish condition
i.c.v.	intracerebroventricular
IgG	immunoglobulin G
IR	immunoreactive
mAChR	muscarinic acetylcholine receptor
mRNA	messenger ribonucleic acid
MWM	Morris water maze
nAChR	nicotinic acetylcholine receptor
NeuN	neuronal nuclei
NGF	nerve growth factor
NGFr	nerve growth factor receptor

NTR	neurotrophin receptor
p75	low affinity nerve growth factor receptor
PB	phosphate buffer
PBS	phosphate buffered saline
PFA	paraformaldehyde
PND	postnatal day

Introduction

Unravelling the influence of neurotransmitters on brain development is of interest to developmental neuroscientists as it may yield a better understanding of neurodevelopmental disorders. In particular, the neurotransmitter acetylcholine may play an important role in the aberrant brain development of children with Down and Rett syndrome (Berger-Sweeney and Hohman, 1997). However, the effect of cholinergic dysfunction on the morphological development of neurons has only been minimally explored.

The present study examines how neonatal cholinergic denervation affects neurons in the hippocampus. As well, it examines the neurobehavioral response to being housed in an enriched environment as this could provide insight regarding how developmental cholinergic dysfunction might alter the ability of the brain to be tuned by the environment. Exposure to an enriched environment during development has positive effects on brain development. It increases the generation of new neurons (neurogenesis), increases synaptic density and the complexity of neuronal branching, and enhances spatial learning (van Praag, Kempermann and Gage, 2000; Kolb et al. 2003).

Cholinergic system

One of the first substances to be identified as a neurotransmitter was acetylcholine (ACh). Primarily known for its function at skeletal neuromuscular junctions, ACh is also found at synapses of the autonomic nervous system and in the central nervous system. The synthesis of ACh occurs mainly in nerve terminals and it is produced from the precursor acetyl coenzyme A, which is synthesized first from glucose and choline. Choline acetyltransferase, the rate-limiting enzyme for the production of ACh, facilitates

the binding of acetyl coenzyme A with choline (van der Zee and Luiten, 1999). Once released from the nerve terminal, ACh binds to its receptors or is rapidly catalyzed by the hydrolytic enzyme acetylcholinesterase (AChE).

Acetylcholine's actions on the postsynaptic terminal are mediated by two types of receptors, the nicotinic receptors (nAChRs) and the muscarinic receptors (mAChRs) (Hosey, 1992). Nicotinic receptors are ion-gated channels (ionotropic) found primarily in skeletal muscles but are also found in the brain, whereas muscarinic receptors are G protein-coupled receptors (metabotropic) and are evident in autonomic ganglia and parasympathetic target organs. They are also abundant in the striatum and other forebrain regions (Shepherd, 1998; van der Zee and Luiten, 1999). Using autoradiography, Lucas-Meunier and colleagues (2003) localized muscarinic receptors in several regions of the brain including the olfactory anterior nucleus, the olfactory tubercle, the hippocampus, the hypothalamic supraoptic nucleus, the nucleus accumbens and the cortex. There are five different types of muscarinic receptor proteins, identified as M1 to M5 (Hosey, 1992; Lucas-Meunier et al. 2003). M1 and M3 are known to be expressed mainly on projecting neurons and M2 and M4 receptors are more highly expressed on interneurons (Cobb and Davies 2005). When activated at presynaptic sites, mAChRs have an inhibitory action by decreasing glutamate release, whereas when present at postsynaptic sites their function is to reduce potassium conductance thereby increasing the likelihood of firing an action potential (Shepherd, 1998). The nicotinic receptors are also abundant in the hippocampus, serving to increase the release of glutamate by allowing calcium entry into the neuron. These receptors also modulate inhibition when present on interneurons (Shepherd, 1998; Wiesner and Fuhrer, 2006).

Forebrain cholinergic projections emanate from the basal forebrain (including the medial septum, the diagonal band of Broca and the nucleus basalis magnocellularis) and innervate the hippocampus and neocortex (Walsh et al. 1995; Lucas-Meunier et al., 2003; Paban et al., 2005). In the rat brain, there are six main projections of the cholinergic forebrain arising from the nuclei Ch1-Ch6 (Lucas-Meunier et al., 2003, see Figure 1). Fibers projecting exclusively to the hippocampus arise from either the cholinergic nuclei of the septum (Ch1) or the vertical limb of the diagonal band (Ch2). The olfactory bulb receives projections from the horizontal limb of the diagonal band (Ch3), whereas the nucleus basalis magnocellularis (Ch4) innervates the cortex. The thalamus receives input from the brainstem pedunculopontinus nucleus (part of Ch5) and the laterodorsal tegmental nucleus (Ch6).

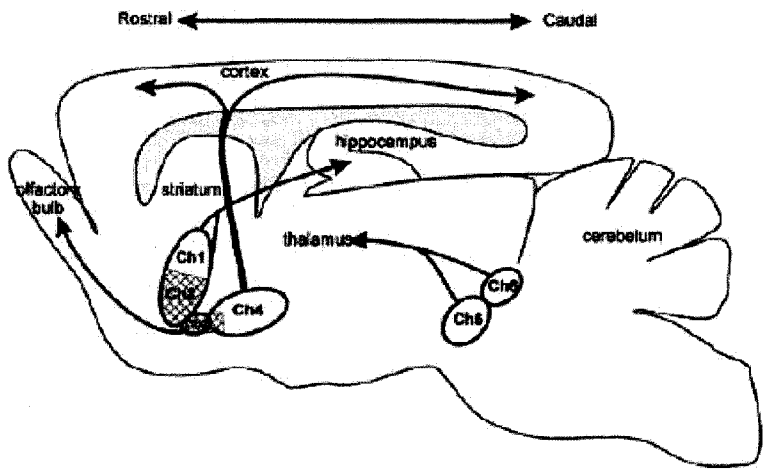


Figure 1. Cholinergic brain systems. From Lucas-Meunier et al., 2003.

The cholinergic basal forebrain has been suggested to be involved in processes such as attention, memory, stress and learning (Ricceri et al. 1999; Lucas-Meunier et al. 2003). It has also been hypothesized to play a critical role in the development of the cortex and cognitive functions (Berger-Sweeney, 2003). Dysfunction in the cholinergic

system may be pathogenic to neurodevelopmental disorders such as Rett and Down syndrome and as well, in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Creutzfeldt-Jakob disease and Korsakoff's syndrome (Lucas-Meunier et al. 2003).

Projections from the medial septal nucleus (30-50%) and the vertical limb of the diagonal band (50-75%) to the hippocampal formation form the total cholinergic input to the hippocampus (Lucas-Meunier et al.; 2003, Shepherd, 1998). That said, lesions of the basal forebrain (medial septum, the diagonal band of Broca and the nucleus basalis magnocellularis) significantly alter the cholinergic input to the hippocampus hence disrupting processes mediated by this region.

Creating Ach lesions using 192-IgG–saporin

Cholinergic basal forebrain neurons express high levels of nerve growth factor receptor (NGFr) coupled to the p75 neurotrophin receptor ($p75^{NTR}$). 192-IgG–saporin (192S) is an immunotoxin containing a ribosome inactivating protein (saporin) conjugated to a monoclonal antibody that has affinity for the $p75^{NTR}$ (Wenk et al. 1994; Sherren, Pappas and Fortin, 1999; Ricceri et al. 1999). The $p75^{NTR}$ is present only in basal forebrain cholinergic neurons, allowing for a selective lesion of only these neurons by 192S (Wrenn and Wiley, 1998; Ricceri et al. 1999; Sherren, Pappas and Fortin, 1999). Following its intraventricular administration, 192S attaches to the $p75^{NTR}$ receptor on the terminal of cholinergic neurons and becomes internalized and transported to the cell body. Accumulation of the toxic saporin moiety in the cell body disrupts functioning of the neurons ribosomal apparatus, leading to complete loss of neuronal function and then death (Leanza et al. 1995).

In both rats and mice, cholinergic basal forebrain fibers enter the neocortex within one week following birth, with hippocampal innervation developing shortly thereafter (Berger-Sweeney, 1998). Sexual dimorphism was also demonstrated to have an effect on the development of the cholinergic system. Full expression of NGFr in the cholinergic basal forebrain neurons may occur earlier in female development compared to males (Berger-Sweeney, 1998; Berger-Sweeney, 2003).

It has been argued that postnatal day 7 is the best age to perform neonatal cholinergic lesioning, as this age coincides with the peak in mRNA expression of $p75^{NTR}$, suggesting a high level of affinity for the 192S and a greater likelihood of creating a more complete lesion of the system (Sherren, Pappas and Fortin, 1999). In fact, intracerebroventricular (i.c.v.) administration of 192S on postnatal day 7 resulted in an 80-90% elimination of basal forebrain choline acetyltransferase (ChAT) and selective, permanent loss of cholinergic innervations of the cortex and hippocampus (Ricceri, Calamandrei and Berger-Sweeney, 1997; Ricceri et al., 1999; Pappas and Sherren, 2003; Pappas et al. 2005; Sherren and Pappas, 2005). The loss is more severe in the hippocampus where there is an over 80% loss of activity of ChAT (Pappas et al, 1996; Sherren and Pappas, 2005).

Behavioural Consequences of neonatal cholinergic lesion

Cholinergic lesions performed on neonates impair adult radial arm maze performance. 192S lesioned animals are moderately slower to accomplish the task (Pappas et al, 1996; 2005). Spatial navigation ability as reflected by Morris water maze acquisition is unaffected although it may be impaired when the animals are old (~ 22 months of age) (Leanza et al., 1996; Pappas et al, 1996; Pappas et al., 2000; Sherren,

Pappas and Fortin, 1999; Pappas et al., 2005). It has also been reported that neonatal cholinergic lesioned animals show impaired acquisition and a deficit in retention on a passive avoidance task (Sengstock et al., 1992; Ricceri, Calamandrei and Berger-Sweeney, 1997; Ricceri, Hohmann and Berger-Sweeney, 2002), exhibit reduced wall rearing on an open field task (Ricceri, Calamandrei and Berger-Sweeney, 1997) and are hyperactive when introduced to a novel object (Ricceri, 2003). However, reports of behavioural deficits in neonatal lesioned animals are not consistent. Hyperactivity after exposure to a novel object was not reported by Leanza and colleagues (1996), no effects were reported on tasks such as object novelty and open field (Ricceri et al., 1997) and neither were any effects of neonatal lesion reported on the elevated plus apparatus by Pappas et al (1996), delayed spatial alternation (Pappas et al., 2000) or a vigilance task (Pappas et al, 2005). Taking into account all the research done on neonatal cholinergic lesioned animals, Ricceri (2003) came to the conclusion that deficits will be noticeable only in high attention demanding tasks involving acquisition of information and long-term memory but not in tasks involving only short-term memory and little demand on attention. On the other hand, Pappas and Sherren (2003) have concluded that the lesion fails to have notable effects on attention, learning and memory. Then again, complex problem solving is compromised. They suggest that this may be further exacerbated with aging.

Neural Consequences of neonatal cholinergic lesion

Neuronal development occurs in three specific stages: neuronal generation (proliferation), migration and differentiation (Berger-Sweeney and Hohmann, 1997). Rapid reproduction and multiplication of cells characterizes the cell proliferation stage,

whereas migration defines the displacement of these cells to other regions, and differentiation is the establishment of new function for existing cells. Disruption at each of these stages can cause respectively, hypoplasia (reduction in cortical cell number), ectopia (abnormal location of neurons), or dysplasia (abnormality in the shapes and number of dendrites) (Berger-Sweeney and Hohmann, 1997).

Accordingly, the administration of 192S to 7-day-old rat pups would be expected if anything, to mainly produce dysplasias in the cortex since proliferation there is complete at this time while migration is nearly complete. Only a handful of studies have addressed the effects of neonatal 192S on neuronal cytoarchitecture and all have focused on the cortex. Hohmann and colleagues (1998) were the first group to report that after neonatal electrolytic lesion of the basal forebrain, morphological abnormality was evident through Nissl staining of pyramidal cells in the fronto-parietal and sensory-motor cortex. This group later investigated pyramidal cells in the somatomotor cortex through rapid Golgi method and demonstrated that changes in apical branching pattern and usual dendritic morphology when comparing ipsilateral to contralateral lesion side (Hohmann et al., 1991). The problem with these studies from Hohmann and colleagues is the lack of specificity of their lesion to cholinergic neurons.

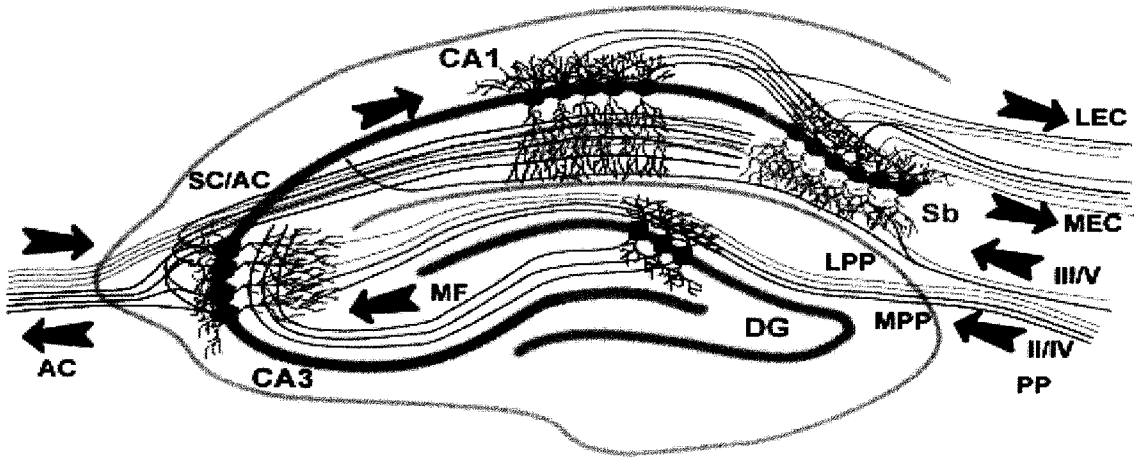
A study using neonatal 192S and retrograde labeling after DiI placement in the superior colliculi reported a reduction in numbers of branches, length and spine density of apical dendrites in the visual cortex of the rat (Robertson et al, 1998). More recently, Sherren and Pappas (2005) used the analysis of Golgi stained neurons to demonstrate that neonatal 192S induced ACh lesion significantly reduced apical branching and basilar dendrites in the retrosplenial cortex and medial prefrontal cortex. Furthermore, this was

observed when the rats were adult, indicating a long lasting if not permanent effect on cortical neuron cytoarchitecture. Dendritic spine density was only affected in the latter area. The hippocampus was not examined, it might be expected to be even more profoundly affected since proliferation and differentiation continue there for several weeks longer and furthermore, the extent of the cholinergic denervation after PND7 192S is greater there than in cortex. Accordingly, in the study reported here, the pyramidal cells in the CA1 region of the hippocampus were chosen to be subjected to cytoarchitectural analysis with the Golgi technique.

Synaptic organization of the hippocampus

As mentioned above, the hippocampus is one of the main targets of the forebrain cholinergic system and possesses both muscarinic and nicotinic ACh receptors. The principal cell groups of the hippocampal formation include the granule cell layer of the dentate gyrus and the pyramidal cell layers in the CA1-CA3 regions. The perforant path, which carries information from the entorhinal cortex and synapses on dentate granule cells, forms the direct cortical input to the hippocampus. Information flow within the hippocampus proceeds from the dentate granule cells to CA3 via the mossy fibre pathway, and from CA3 to CA1 in the Schaffer collateral projection. The input from the CA3 fibers is normally onto CA1 apical tree. Finally CA1 pyramidal cells project to the entorhinal cortex (Shepherd, 1998). The granule cells are defined as polymorphic, implying that they are of various types that project to other part of the dentate gyrus as well as conveying fibers to the CA3 region (Shepherd, 1998). The hippocampal pyramidal neurons (CA1-CA3) have large dendritic trees projecting in both directions, classifying them as multipolar neurons. The apical trees originate from the cell body and

project toward the center of the hippocampus i.e. the dentate gyrus, whereas the basal trees originate from the cell body and project into the stratum oriens and towards the cortex. The arborization of the apical tree is normally longer than the basal tree (Shepherd, 1998). Most dendrites of the pyramidal neurons possess spines, which are classified as standard "cortical-like" spines and are able to form excitatory synapses (Shepherd, 1998). Figure 2 (http://www.gnxp.com/blog/archives/2007_01_01_gene-expression_archive.php) schematizes the hippocampal network.



The Hippocampal Network: The hippocampus forms a principally uni-directional network, with input from the Entorhinal Cortex (EC) that forms connections with the Dentate Gyrus (DG) and CA3 pyramidal neurons via the Perforant Path (PP - split into lateral and medial). CA3 neurons also receive input from the DG via the mossy fibres (MF). They send axons to CA1 pyramidal cells via the Schaffer Collateral Pathway (SC), as well as to CA1 cells in the contralateral hippocampus via the Associational Commissural pathway (AC). CA1 neurons also receive input directly from the Perforant Path and send axons to the Subiculum (Sb). These neuron in turn send the main hippocampal output back to the EC, forming a loop.

Enriched environment

Housing conditions can have a tremendous effect on cellular, molecular and behavioural aspects of brain function. The idea of an enrichment effect on brain and behaviour goes back to 1791 when a scientist by the name of Malacarne was interested in comparing the brains of his dogs and birds reared in either a training/enriched or isolated

environment (cited by Rosenzweig, Bennett and Diamond, 1972). Hebb (1947) was the first to define enrichment in a neuroscientific context and to publish an experiment on enrichment. He noticed that the rats he kept as pets at home (enriched condition) show "definite superiority" over the group raised in laboratory cages on the Hebb-William test method (Hebb, 1947). In today's neuroscience context, environmental enrichment is usually defined as housing conditions that facilitate exploration, enhance sensory, cognitive or motor stimulation or include some physical activities (van Praag, Kempermann and Gage, 2000). Practically, enrichment usually involves a large home cage and objects that stimulate the animal's exploration. These are usually changed on some regular schedule. As well, behavioural tasks that include learning and memory training, where the animal is exposed to a novel environment, are sometimes also applied. Some of the major behavioural effects resulting from enrichment are enhanced learning and memory, decreased anxiety and increased exploratory activity (Nithianantharajah and Hannan 2006). Memory (specifically spatial memory) in several learning tasks is notably enhanced by enrichment. Animals exposed to enrichment perform better on complex maze tasks such as the Hebb-Williams maze and on simpler spatial learning procedures such as the Morris water maze and the T-maze (Brown and King, 1971; Will and Rosenzweig, 1976; Kelche and Will, 1978; Kolb and Whishaw, 1998; van Praag, Kempermann and Gage, 2000; Paban et al., 2005).Environmentally enriched animals also showed decreased anxiety-like behaviour compare to control animals when assessed on elevated plus maze by spending more time in the open arms. They also expressed less freezing behaviour on stress paradigm demonstrating valuable enrichment effect on anxiety-like behaviour (Chapillon et al., 1999; Benaroya-Milshtein et al., 2004).

Enrichment can also increase neural activation, signaling and plasticity in several brain regions such as the somatosensory and visual cortices as well as the hippocampus, motor cortex and cerebellum (van Praag, Kempermann and Gage, 2000; Nithianantharajah and Hannan 2006). At the neural level, enriched environment was first found to result in increased weight and thickness of cortex (Rosenzweig et al., 1962; Bennett et al., 1964; West and Greenough, 1972; Cummins et al., 1973; Uphouse, 1980; Brenner et al., 1985; Kolb and Whishaw, 1998). Subsequently, it was discovered that this was not due to increased neurons but rather increase dendritic branching and dendritic length, and dendritic spines, particularly in the somatosensory cortex (Kolb et al., 2003). Analysis of Golgi stained brain sections has been particularly instructive in this regard (Greenough and Volkmar, 1973; Greenough, Volkmar and Juraska, 1973; Fiala, Joyce and Greenough, 1977; Uylings et al., 1978; Green, Greenough and Schlumpe, 1983; Kolb and Whishaw, 1998; Faherty, Kerley and Smeyne, 2003; Kolb et al., 2003)

Increased gene expression of nerve growth factor (NFG), brain-derived neurotrophic factor (BDNF) and glial-cell derived neurotrophic factor (GDNF) also result from exposure to enriched environments (Ickes et al., 2000). An increased in the amount of acetylcholinesterase in the brain was also reported in enriched animals (Faherty, Kerley and Smeyne, 2003).

One goal of the present study was to determine if neonatal lesioning of the basal forebrain Ach neurons by 192S would alter the ability of hippocampal pyramidal neurons to be shaped by enrichment. Only few studies have looked at the hippocampal pyramidal neurons. Like cortical pyramidal cells, the hippocampal pyramidal neurons are influenced by enrichment. CA1 pyramidal cells demonstrate morphological changes due to

enrichment such as increased number of branches and dendritic length (Barlesaghi, Severi and Guidi, 2003; Faherty, Kerley and Smeyne, 2003). Since our laboratory has observed that the neonatal 192S lesion reduces the synaptic complexity of cortical neurons (Sherren and Pappas, 2005), its effects on CA1 neurons and in particular its effects on the response of these neurons to enrichment was of interest. Previous studies showed that the hippocampus is densely innervated by cholinergic fibers from the medial septum and the vertical limb of the diagonal band of Broca. These innervations form a dense fiber network connecting to the granule and molecular cell layer (Clarke 1985; Aznavour, Watkins and Descarries, 2005; Mohapel et al., 2005).

It has also recently been noted that enriched environment increases neurogenesis in the adult hippocampus (van Praag, Kempermann and Gage, 2000; Nithianantharajah and Hannan 2006). Neurogenesis in the adult brain was first mentioned in reports of the formation of new neurons in such structures as the hippocampus and the olfactory bulb in the adult brain of rat and cat (Altman, 1962; Altman and Das, 1965; Altman and Das, 1966; Altman, 1967; Cooper-Kuhn, Winkler and Kuhn, 2004). Using tritiated thymidine, which is incorporated into the DNA of cells undergoing replication, Altman and co-workers observed accumulation of reduced silver grains consistently over the granule cells of the hippocampus and over the nuclei of few neurons in the neocortex in adult rats suggesting multiplication of these cells, hence neurogenesis (Altman, 1962). Curiously, regardless of the importance of this discovery, adult neurogenesis was ignored for many years before another group of researchers (Kaplan and Hinds, 1977) looked at the issue again. It is now well established that neuronal progenitor cells are produced in the subgranular zone of the dentate gyrus and then migrate to the granule cell layer for

differentiation, resulting in the formation of new neurons or glia. The newly generated cells can thereafter establish functional connections in the CA3 and CA1 region of the hippocampus when they reach maturity (Mackowiak et al, 2004). Recent studies indicate that the rate of neurogenesis can be controlled by both intrinsic factors (including genetic makeup, age, growth factors, hormones and neurotransmitters) and external factors (such as environmental or pharmacological stimuli) (Mackowiak et al., 2004). These factors can interact with neurogenesis by either increasing or decreasing its rate. One of the most powerful intrinsic modulators of neurogenesis is advanced age, which causes a substantial decline. With respect to extrinsic modulators, enrichment has been shown to increase neurogenesis in the dentate gyrus and to influence dendritic maturation and cortical differentiation (Mackowiak et al, 2004).

Of interest in the present study is the effect of enrichment on neurogenesis and how this might be modulated by cholinergic lesion. Given that neonatal cholinergic basal forebrain lesions very substantially reduced cholinergic innervation of the hippocampus, and that the dentate gyrus is one of the two regions where neurogenesis occurs in the adult brain (Cooper-Kuhn, Winkler and Kuhn, 2004), the likelihood that cholinergic lesion have an impact on neurogenesis is reasonable. The dentate gyrus receives abundant cholinergic innervation from the medial septum and lesion there profoundly affects the cholinergic hippocampal network (Clarke, 1985; Aznavour, Watkins and Descarries, 2005; Mohapel et al., 2005). A recent study showed that cholinergic denervation in the adult rat results in a decline in the generation of new neurons in the granule cell layer of the hippocampus, suggesting that acetylcholine modulates the fate of progenitor cells of the dentate gyrus and perhaps the olfactory bulb as well (Cooper-Kuhn, Winkler and

Kuhn, 2004). The influence of neonatal cholinergic lesion on DG neurogenesis has not been examined. It was one of the main objectives of the present study.

Summary and Research Objectives

The overriding objective of this project was to determine if neonatal forebrain Ach lesion would alter the effects of enrichment on

- Behaviour as reflected by Morris water maze performance
- CA1 cell cytoarchitecture as illuminated by the Golgi stain
- Dentate gyrus neurogenesis as reflected by immunostaining for doublecortin, a useful marker of newborn neurons

As mentioned, previous research found neonatal cholinergic lesions resulted in little if no impairment of spatial learning in the Morris water maze when the rats were tested as adults. Furthermore, enrichment improves performance of the Morris water maze in normal rats (Nithianantharajah and Hannan 2006; van Praag, Kempermann and Gage, 2000). The present experiment determined if this effect of enrichment is also evident in neonatal cholinergic lesioned rats.

As previously mentioned, both of neonatal Ach lesion and enrichment affect neuronal cytoarchitecture. Their combined effects have not been examined. Since neonatal 192S reduces the complexity of dendritic branching of cortical pyramidal neurons (Sherren and Pappas, 2006) while enrichment increases dendritic branching and complexity, the effects of combining this lesion and enrichment warrant examination. Would the lesion abolish the response to enrichment? Conversely, would enrichment normalize dendritic structure in lesioned animals? Golgi-Cox staining was used to quantitatively characterize pyramidal neurons. The Golgi-Cox staining is a very useful

technique for staining entire processes of single neurons (Frostscher, 1992; Kolb and Wishaw, 1998). This technique allows for reliable random staining, permitting the visualization and estimation of dendritic trees and synaptic spines of pyramidal neurons (Kolb and Whishaw, 1998). The Golgi-Cox procedure was used here to estimate the number of dendritic spines and dendritic length of neurons in the area of interest. Pyramidal neurons are the most common type of cells found in the cortex and their dendritic spines are responsible for receiving the majority of synaptic input. Pyramidal neurons are also present in the CA1 region of the hippocampal formation.

Lastly, as briefly reviewed earlier, enrichment promotes neurogenesis in the dentate gyrus while adult forebrain cholinergic lesions reduce neurogenesis. The present experiment examines the effect of neonatal forebrain cholinergic lesion on the ability of enrichment to stimulate neurogenesis. To investigate neuron proliferation in the dentate gyrus the neuronal marker doublecortin (DCX) was chosen. DCX is a marker of proliferation of cells committed to neuronal phenotype, i.e. newborn neurons (Christie and Cameron, 2006; Kempermann et al., 2004; Koizumi et al., 2006; Plumpe et el. 2006; Rao and Shetty, 2004; Brown et al., 2003; Couillard-Despres et al., 2005) in contrast to commonly used marker bromodeoxyuridine (BrdU) which is not specific to neurons (newborn cells).

It was our hope that not only would these inquiries provide information about the role of Ach in rat brain and its response to enrichment, but it would also provide hints regarding how cholinergic dysfunction might contribute to the aberrant brain development and cognitive disturbance in human neurodevelopmental disorders such as Down and Rett syndrome, which are characterized by cholinergic dysfunction.

Methods

Animals

Eight female and four male Sprague-Dawley rats were obtained from Harlan (Indianapolis, Indiana) with the intention of using them as breeders. They were single housed in a plexiglass cages (44 x 24 X 20 cm) and maintained on a 12 hour reversed light/dark schedule with free access to water and rat chow. After one week of acclimation (quarantine) to the vivarium, two females were housed with one male until the females showed obvious signs of pregnancy. Only then were the rats separated and single housed for birthing. The day of birth was designated as P0 and the litters were then culled to 8 males at P4 or P5. The lesions were administered on P7 after which the pups were returned directly to their home cage. This breeding procedure was repeated a second time, one week after the pups were weaned, at P21-P23. A total of 16 litters were used and the litter was split and assigned randomly to the different treatment groups.

Surgery

On P7, the pups were subjected to forebrain cholinergic lesion by infusion of the neurotoxin 192-IgG-saporin into the lateral ventricles (192S; Advanced Targeting Systems, San Diego, CA), or they received a sham lesion (vehicle injection only). In total, 56 males received injections whereas 48 males received vehicle injections.

192S was diluted to a concentration of 0.2 μg/μl using 10mM phosphate buffered saline, pH 7.2 (PBS) and stored in aliquots at –80°C. On the day of surgery, 192S was taken out of the freezer and stored at 4°C, allowing it to thaw. The solution was always used within 48 hours after thawing. PBS was used as the vehicle solution.

Half of each litter (4) was removed from the home cage for surgery and returned only when all 4 pups had regained consciousness. The other half was then taken through the same procedures and returned later to the mother. The pups were carefully monitored after surgery to ensure the mother did not reject them.

The induction of anaesthesia was performed using a large nose cone delivering a mixture of isoflurane/oxygen, which was gradually increased up to 4 %isoflurane. Once the toe and tail pinch reflexes were lost, the pups were transferred to a mold mounted on a stereotaxic frame and attached to a small nose cone delivering anaesthetic. The mixture of isoflurane/oxygen was then reduced gradually to 1.5 % and maintained at that level throughout the surgical procedure.

First, a small hole was punched in each animal's left or right ear to provide a permanent means of distinguishing control and lesioned rats, and then a midline incision was made with a scalpel to expose the skull. The location of bregma was then determined and two holes were drilled at ± 1.8 mm lateral to bregma with an electrical drill (Dremel, National Tool Warehouse, Carthage, MO) and surgical bit. A 5µl microsyringe (Hamilton, Reno, NV) with a 30-gauge needle was slowly lowered into each hole 3.5 mm from the dura, aimed at the lateral ventricles. 1.5µl of either 192S or PBS was slowly infused into each lateral ventricle over a period of 2 minutes. An additional 2 minutes was necessary to allow complete diffusion of the solution away from the injection site. The needle was then retracted slowly to prevent any toxin drawing back into the needle tract. Tissue adhesive (Vetbond 3M Worldwide, World Precision Instruments, Sarasota, FL) was used to close the incision site and allow proper healing. During surgery, the pups were placed under a lamp to prevent heat loss, and at all times when the pups were away

from the mother (before and after surgery), the cage was placed on a heating pad set at medium heat. A subset of animals was assigned to behavioural testing whereas the others were killed after the 6 weeks of enriched/standard environment for morphological analysis.

Housing

Weaning occurred between P21 and P23, and the rats were then placed either in standard or enriched housing. The two injection groups were assigned to the two different types of housing, forming 4 groups: 192S + enriched environment (n=29, group 192S-E), 192S + standard environment (n=27, group 192S-I), vehicle + enriched environment (n=28, group PBS-E) and vehicles + standard environment (n=20, group PBS-I). The standard housing consisted of placing two rats from the same treatment group in a plexiglass cages (44 x 24 X 20 cm). The enriched housing consisted of large (72 x 72 x 46 cm) stainless steel cages with wire mesh siding and a plexiglass front panel in which 4-5 same group rats were placed. The enriched cages contained an arrangement of toys (i.e. balls, chains, ladders, tunnels, running wheels, etc.). The rats were exposed to a different arrangement of toys each week when they were moved to a new enrichment cage, and in addition, a new toy was introduced into the cage each day (and an old toy removed). Rats in the enriched housing condition were kept in a separate room from rats in the standard housing condition. The animals stayed in their assigned housing type for 42 days (6 weeks). After this six weeks period, the rats were individually housed in plexiglass cages.

Morris Water Maze (working memory version)

The Morris Water Maze was used to test spatial learning/memory in a subset of the rats after 6 weeks of standard or enriched housing. The working memory version of this task (Whishaw, 1985) was used. In this version, the location of the submerged platform varied from day to day. Training began the day following the end of the enriched/standard environment at approximately 3 months of age. All four groups were tested resulting in 17 rats in the 192S-E group, 14 rats in the 192S-I group, 16 rats in the PBS-E group and 11 rats in the PBS-I group. The maze consisted of a white polypropylene pool fitted with a black insert 1.6 m in diameter and 81 cm in height, filled with room temperature water ($21 \pm 1^{\circ}C$). The room contained large black visual cues on the walls to facilitate spatial mapping. The rats were given 5 trials per day for a period of 5 consecutive days. A transparent plexiglass platform was placed 2 cm below the water level at 12 inches from the wall of the pool. The location of the platform was chosen randomly and it was the same for each animal on that day. On each trial, the rat was started from a different point on the perimeter of the maze but the order of the start positions was consistent across all animals. The rats were given 90 seconds to find the platform and an additional 30 seconds on the platform. If the platform was not located within 90 seconds, the experimenter guided the animal to the platform. The sessions were recorded using an overhead video camera coupled to a video recorder and a computer running SMART tracking software (San Diego Instruments, San Diego, CA). The latency, and path length to find platform as well as swim speed were recorded.

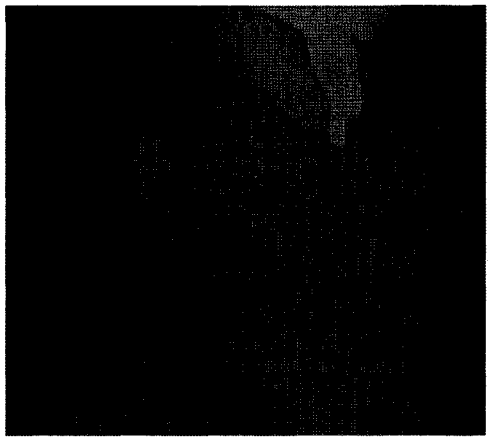
Histological methods

The cohort of animals (n= 39) used for the morphological analysis was killed within 1 day after the end of enriched/standard environment condition. They were injected with sodium pentobarbital overdose and perfused with 200 ml of 0.9% saline. The brains were removed and cut at the midline to separate the two hemispheres. The left hemispheres were placed in 20-30ml of 4% paraformaldehyde (PFA) in 0.1M phosphate buffer, pH 7.4 (PB) at 4°C for 24 hours. The following day, the 4% PFA solution was discarded and the brains were rinsed twice in PB with 0.01% sodium azide. The first rinse was for 4 hours and the second rinse was overnight. The brains were then sectioned in 100 μm saggital sections throughout the whole hippocampus using a vibratome (World Precision Instruments, Sarasota, FL). Sections were collected in PB or in a cryoprotectant solution for long-term storage. The right hemispheres were placed in Golgi-Cox solution (Glaser & van der Loos, 1981) for 14 days in the dark. After the 14 days, the Golgi-Cox solution was replaced with 30% sucrose and placed in the dark for another 2 days. Coronal 200 μm sections were then cut with a vibratome and mounted onto 2% gelatin coated slides. The staining was done following the protocol of Gibb and Kolb (1998).

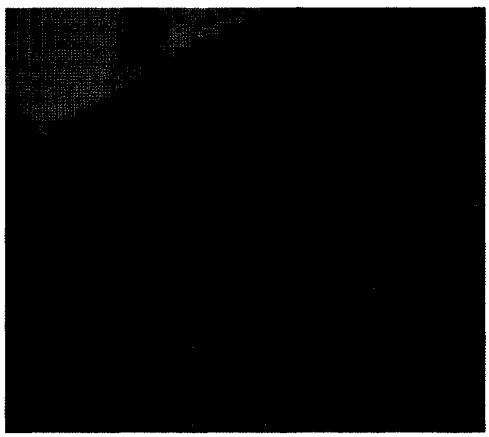
The cohort of animals (n=58) used for behavioural testing was killed on the sixth day. The animals were injected with a sodium pentobarbital overdose and perfused with 75 ml of 0.9% saline and 200 ml of 4% PFA in 0.1M PB, pH 7.4. Following the first 24 hours, the 4% PFA was replaced by 0.1M PB plus azide, pH 7.4 and rinsed twice more at 4 hour intervals. These brains were then sectioned using a vibratome into coronal sections of 100 μm. All histological measurements were performed with the experimenter blind to the group the animal belong.

To confirm the lesions, the left side of every brain from the morphological cohort and the whole brains of the behaviour cohort were stained with p75 neurotrophin receptor monoclonal antibody (Chemicon International, Temecula, CA) following standard immunohistochemistry staining protocol for floated sections. The left side of every brain from the first group of animal and the whole brains from the second group of animals were also stained for acetylcholinesterase using slight modifications of the method of Paxinos and Watson (1998). The sections were first rinsed with sodium acetate then placed in a reaction solution containing sodium acetate, cupric sulfate, glycine, distilled water and acetylthiocholine iodide which is light sensitive. The sections in the reaction solution were placed in a drawer away from the light overnight. On the following day, the sections were first rinsed in sodium acetate, put into sodium sulphide and then rinsed again in sodium acetate and mounted on gelatinized slides for quantification using freely available NIH densitometry software (Scion Image). Finally the left side of every brain from group 1 and the whole brain from group 2 was also used for doublecortin staining using the standard immunohistochemistry for floated sections protocol. Those sections were later quantified using Stereo-Investigator software (Micro-Brightfield Inc, Williston, VT).

One section per brain was stained for p75 neurotrophin receptor and analysis of these sections was done through a comparison using vehicle treated animals as the standard. The rating scale used to quantified the lesioned animals was graded on a 4 points scale where 0 = no cell, 1 = slight number of cells, 2 = moderate number of cells and 3 = same number of cells as vehicle treated animals (represented in Figure 3).



A section from 192S animal showing a severe loss of p75 immunoreactive cells in the vertical limb of the diagonal band. This section was rated as 0/3 on the 4 points scale



A section from PBS animal showing a no loss of p75 immunoreactive cells in the vertical limb of the diagonal band. This section was rated as 3/3 on the 4 points scale

Figure 3. Representation of rating scale for p75 neurotrophin receptor staining

A total of 55-60 sections resulted from slicing a brain through the whole hippocampus. Four sections per brain were utilized for acetylcholinesterase staining, and quantification using densitometry. The densitometry was carried out on the hippocampal region of the brains. We systematically selected 1 section from each of 1-4th quarter of all the sections covering the dorsal hippocampus. The quantification included 3 areas of CA1, one of CA2, CA3, CA4 as well as 2 areas of the DG as shown in figure 4. This densitometry software quantified the density of the staining in all these areas.

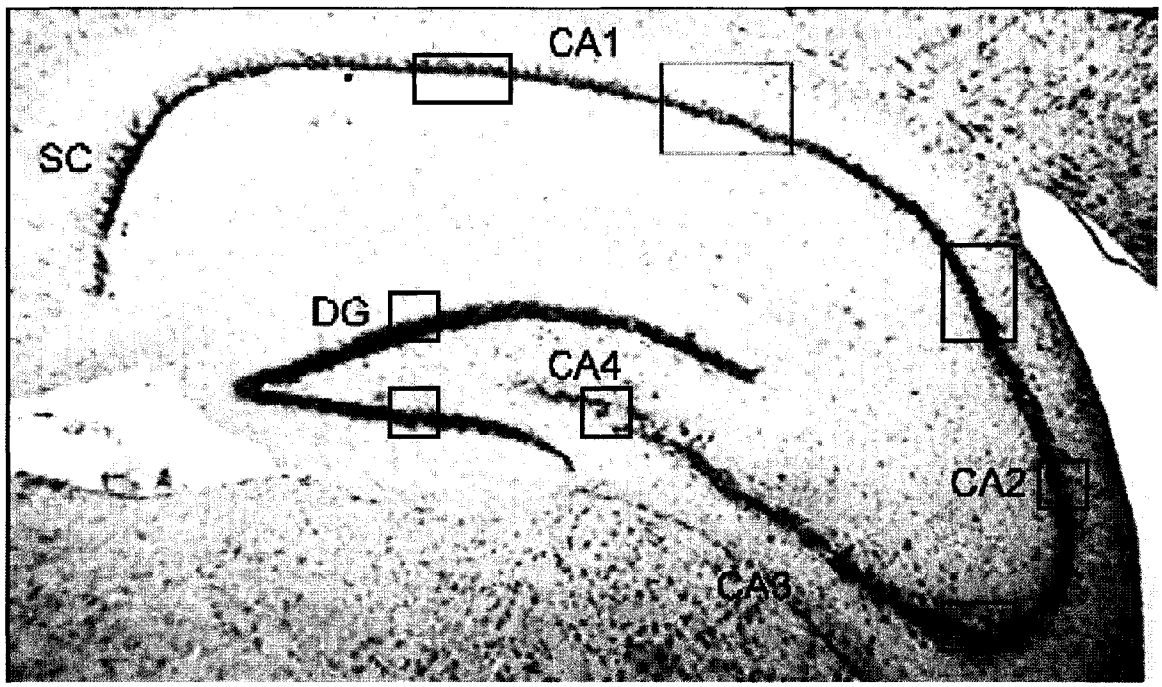


Figure 4. Li, Zhang and Sima, 2005

Finally a set of 10 sections per brain containing the hippocampus was used for doublecortin (DCX) staining. One the first day of staining, the sections were rinsed in PBS + azide twice, incubated in 0.3% H2O2 for 30 mins, rinsed another three times, incubated for 30 mins in 3% normal horse serum, rinsed another three times and incubated overnight in the primary DCX antibody (Santa Cruz Biotechnology Inc, Santa

Cruz, CA) to a concentration of 1:200. The next day of staining, the sections were first rinsed three times in PBS + azide and incubated in antigoat biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) of concentration 1:100 for 2-3 hrs. After the 2-3 hrs, sections were rinsed another three times in PBS no azide and then incubated for another 2-3 hrs in tertiary complex (Amersham Biosciences Inc, Piscataway, NJ) concentration 1:100. Following incubation, section were rinsed in PBS no azide, a DAB reaction was performed and the sections were let to dry overnight. On the third day of staining, the sections were rinsed in dH2O for 2 mins, counterstained with pyronin Y, rinsed in running tap water for 2 mins, and finally dehydrated, cleared, and mounted. The first section was selected at random from the first 10 sections cut, and the following sections were selected at regular intervals (every tenth section). DCX - immunopositive cells in the dentate gyrus were stereologically counted using Stereo-Investigator software (Micro-Brightfield Inc, Williston, VT). This software makes use of the optical fractionator method consisting of a three-dimensional probe known as the optical dissector to estimate the total number of cells in a defined area. All analysis for densitometry, p75 and DCX staining was conducted using a microscope Olympus BX 51.

The Golgi analysis was restricted to the male rats that had not been subjected to behavioural testing. The brain structure analyzed was chosen based on existing anatomical literature and consisted of the CA1 pyramidal cell layer of the hippocampus. The entire postsynaptic dendritic field of each selected pyramidal cell was traced using the Neuroleucida software and an Olympus BX51 microscope, viewed with the 100X objective. Cells were selected from the optical planes located in the middle third of the tissue depth such that the apical and basal trees were contained entirely within the 200

μm slice, and were not heavily obscured by blood vessels or dense clusters of dendrites from neighbouring cells. Data were collected from the apical and basal dendritic trees and averaged for the ten cells per dorsal hippocampal CA1 per rat.

The estimation of the total dendritic length, branching and spine density in both apical and basal trees of the traced cells were performed using Neuroleucida Explorer software. The spine density was determined from both the apical tuft and basal tree using the 100X objective and expressed as a density measure. The spine density estimate likely underestimated the true spine density, as spines located either above or beneath the dendrite are not visible with this method.

Results

General health after 192S Lesion

There were no deaths related to the surgical procedure in this study. The animals underwent neonatal cholinergic lesion or sham operation on postnatal day 7 and all of them survived for the duration of the study. The general health of the animals was monitored post-surgery and there was no evidence to indicate that any pups suffered from maternal neglect or impaired ability to obtain food from the mother. The animals body weight was taken at time of perfusion and the group of animals that was sacrificed immediately after enrichment/standard housing showed a significant difference for the housing condition ($F(1,35) = 10.955$ $p<0.002$) but not for the lesion condition ($F(1,35) = 0.952$ $p<0.336$). The animals kept in enrichment were lighter compared to the animals kept in standard housing most likely as a result of the increased activity and exercise associated with enriched environment. The second group of animals that was sacrificed after water maze testing did not show any significant effect of body weight on either the lesion ($F(1,60) = 2.20$ $p<0.14$) or the housing condition ($F(1,60) = 0.02$, $p<0.89$).

Assessment of 192S Lesion

p75 Immunohistochemistry. Tissue sections containing the nucleus of the horizontal and vertical limb of the diagonal band stained for p75 neurotrophin receptor ($p75^{NTR}$) were analyzed by comparison to vehicle treated animals as the standard. The rating scale used to quantify the $p75^{NTR}$ staining was a graded 4 points scale where 0 = no $p75^{NTR}$ immunoreactive (IR) cells, 1 = slight number of cells, 2 = moderate number of cells and 3 = same number of cells as vehicle treated animals. Any 192S lesioned animal

that was graded with a score of 2 or 3 was discarded from the study due to incomplete lesion of the cholinergic system.

Four tissue sections per brain containing the hippocampus stained for acetylcholinesterase were quantified using densitometry. The areas CA1, CA2, CA3, CA4 and the dentate gyrus of the hippocampus were analyzed and averaged for all four sections of each brain. Any 192S lesioned animal that showed an average percentage density equal to the average of the control group was also discarded from the study due to incomplete lesion. As it turned out, the brains discarded from the $p75^{NTR}$ staining were also discarded from the densitometry analysis, showing consistency in these two criteria. Figure 5 shows a photomicrograph of acetylcholinesterase representing the hippocampus and $p75^{NTR}$ staining in the vertical limb of the diagonal band of a PBS animal, a 192S animal with complete lesion and a 192S animal with an incomplete lesion. Of the total of 104 male rats used for this experiment, 7 animals (4 from 192S-E and 3 from 192S-I) were discarded due to incomplete lesion. As a result, 58 male rats were used for Morris water maze testing and 39 male rats were processed for Golgi-Cox staining.

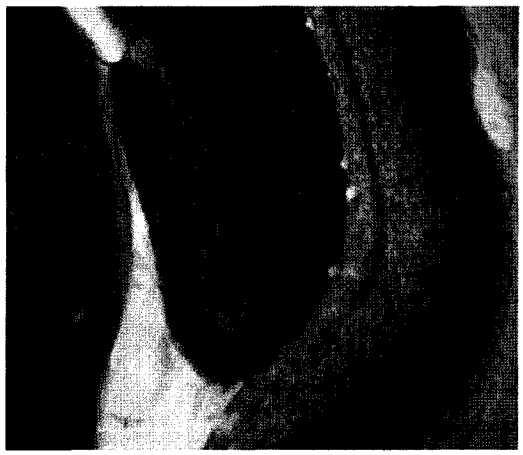
Morris Water Maze

The number of rats assigned to this task consisted of 17 rats in the 192S-E group, 14 rats in the 192S-I group, 16 rats in the PBS-E group and 11 rats in the PBS-I group. A repeated measure ANOVA was performed to analyze the data. The within subjects factor was the 5 test days and the between-subjects factors were the housing condition and the lesion treatment. Animals received 5 daily trials for five days where latency, distance and

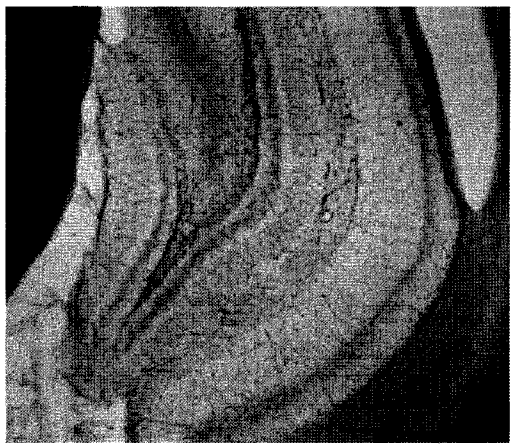
Figure 5. Photomicrograph of acetylcholinesterase (left) representing the hippocampus and p75 ^{NTR} (right) staining representing the vertical limb of the diagonal band. Top two pictures represent a PBS animal with no lesion, the middle two pictures represent an 192S animal with complete lesion and the bottom two pictures represent an 192S animal with an incomplete lesion.

Acetylcholinesterase

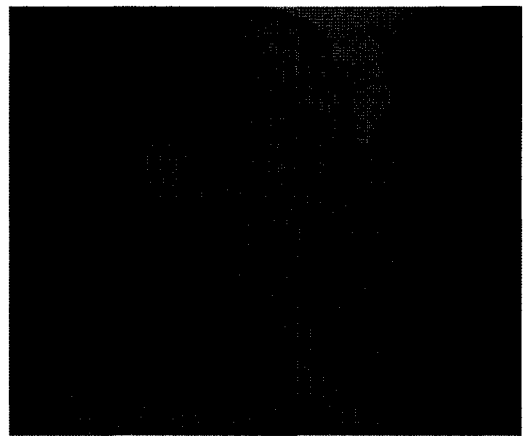
$p75^{NTR}$

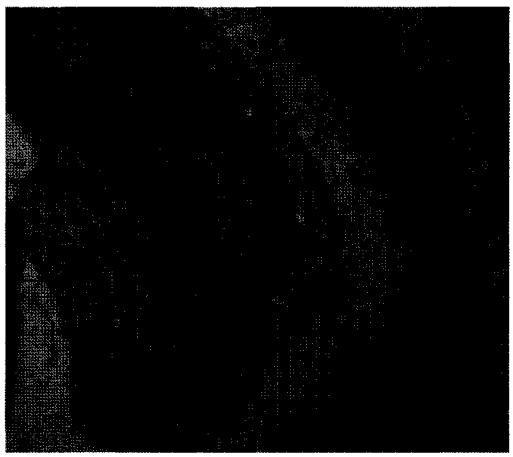


PBS,
no lesion

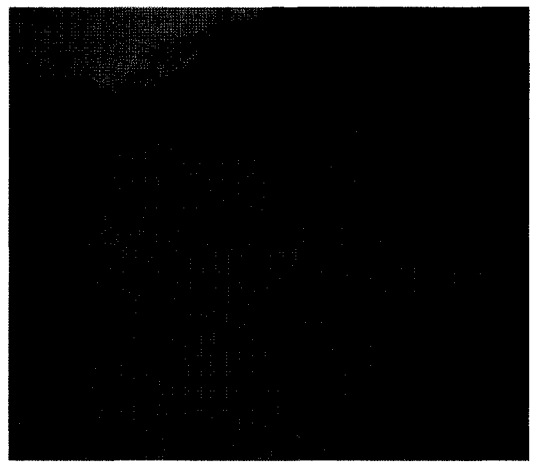


192S,
good lesion





192S,
partial lesion



swim speed to find the hidden platform were recorded for each swim. The latency, distance and swim speed from the last four trials were averaged for each daily session (trial one was not included as on this trial, the new platform location was discovered).

Figure 6 shows the average daily scores for distance travelled to locate the platform. Figure 7 shows the average daily scores for latency until discovery of the platform. Figure 8 shows the average daily scores for speed to reach the platform.

A significant effect of the lesion condition was found for the latency measure ($F(1,54) = 7.53$ $p<0.008$) while the effect for distance fell short of significance ($F(1,54) = 2.82$, $p<0.099$). Overall, the 192S group was significantly slower to find the platform than the PBS group. A day by day analysis showed that the lesion effect was significant on day 3 ($p<0.007$) and day 5 ($p<0.001$). Day 4 just missed significance ($p<0.059$). A significant effect of housing condition was seen on both latency ($F(1,54) = 43.66$, $p<0.000$) and on distance ($F(1,54) = 45.98$, $p<0.000$). The enriched environment groups performed significantly better on both latency and distance measures. Additional ANOVAs incorporating only the data for each day showed that the enrichment effect was significant on days 1-4 but not day 5 for both distance and latency.

The interaction between lesion and housing conditions was not significant for either the latency ($F(1,54) = 1.669$, $p<0.202$), distance ($F(1,54) = 0.072$, $p<0.790$) or speed ($F(1,54) = 3.500$, $p<0.067$) indicating that the performance enhancing effect of the enriched housing condition did not differ for the PBS and the 192S groups. This was confirmed by separate ANOVAs for the PBS and 192S groups, which showed significant

Figure 6 Morris water maze, working memory version. The graph shows the average daily scores for distance travelled to locate the platform. There was a significant effect for the housing condition ($F(1,54) = 45.98$, $p<0.000$) and neither a lesion nor a housing by lesion effect.

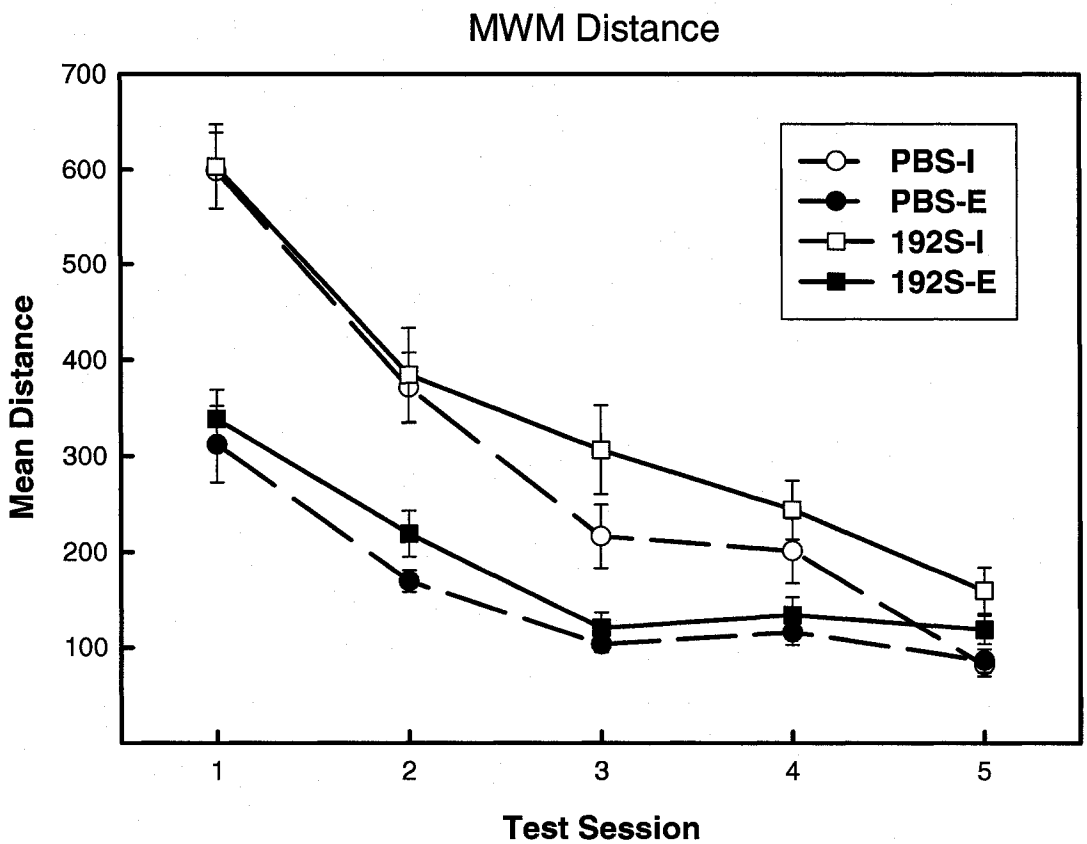


Figure 7. Morris water maze, working memory version. The graph shows the average daily scores for latency until discovery of the platform. There is a significant effect for the housing condition ($F(1,54) = 43.66$, $p<0.000$) and lesion condition ($F(1,54) = 7.53$, $p<0.008$). The interaction was not significant.

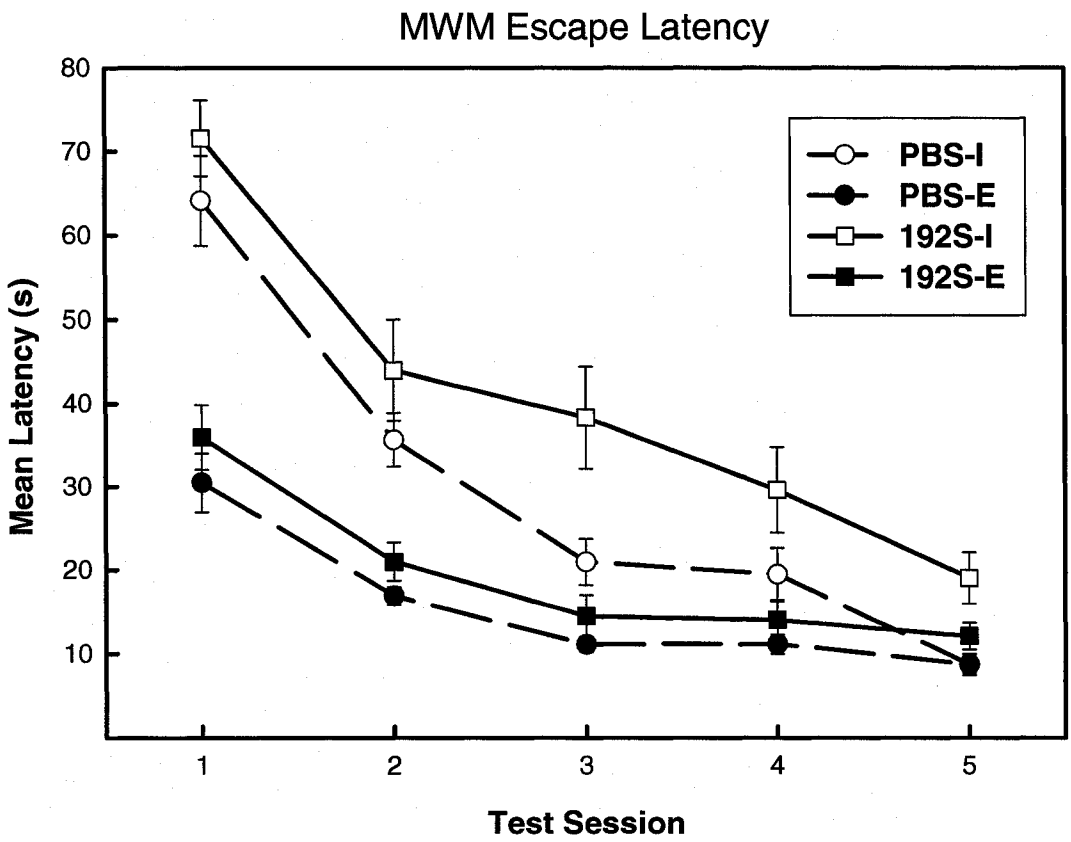
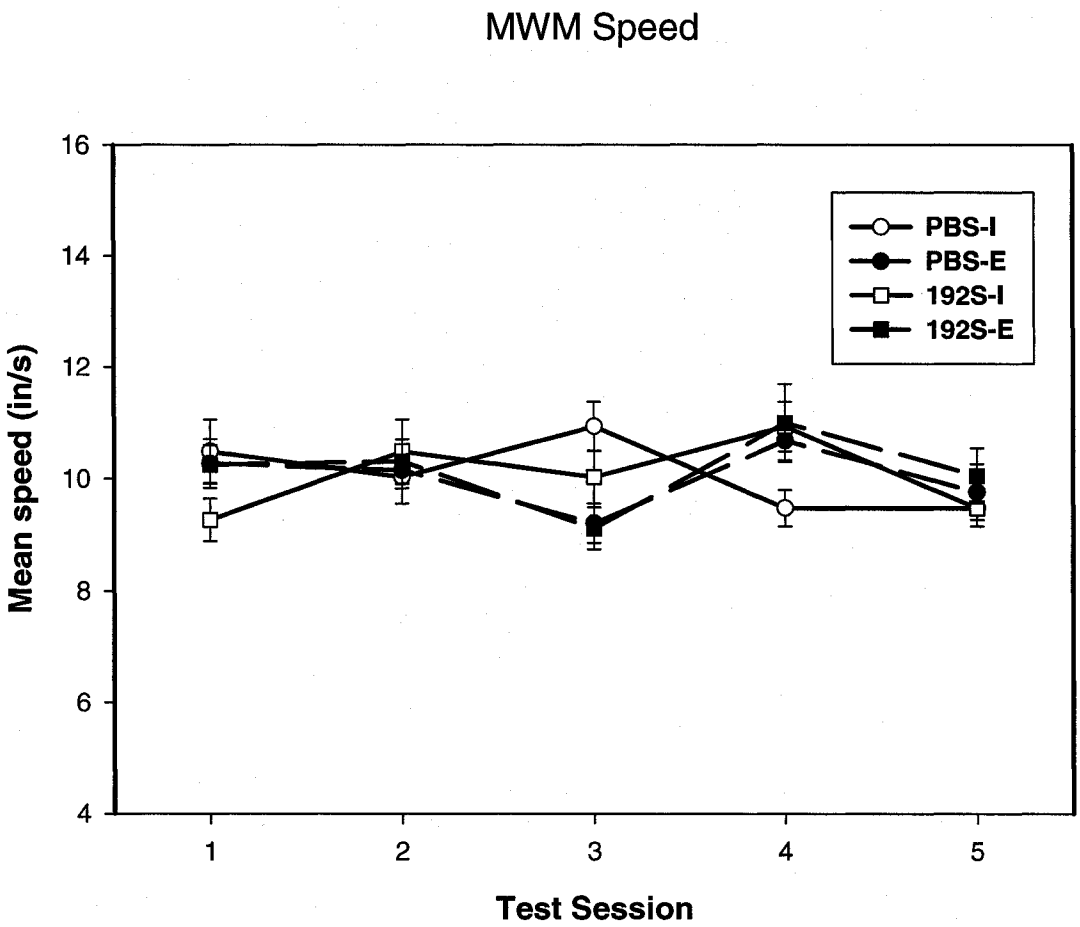


Figure 8. Morris water maze, working memory version. The graph shows the average daily scores for speed to reach the platform. There is a significant effect for the lesion condition ($F(1,54) = 4.49$, $p<0.039$). No significant effect for the housing condition.



effects of the enrichment condition on both the latency, and distance measures (all p values were less than 0.000).

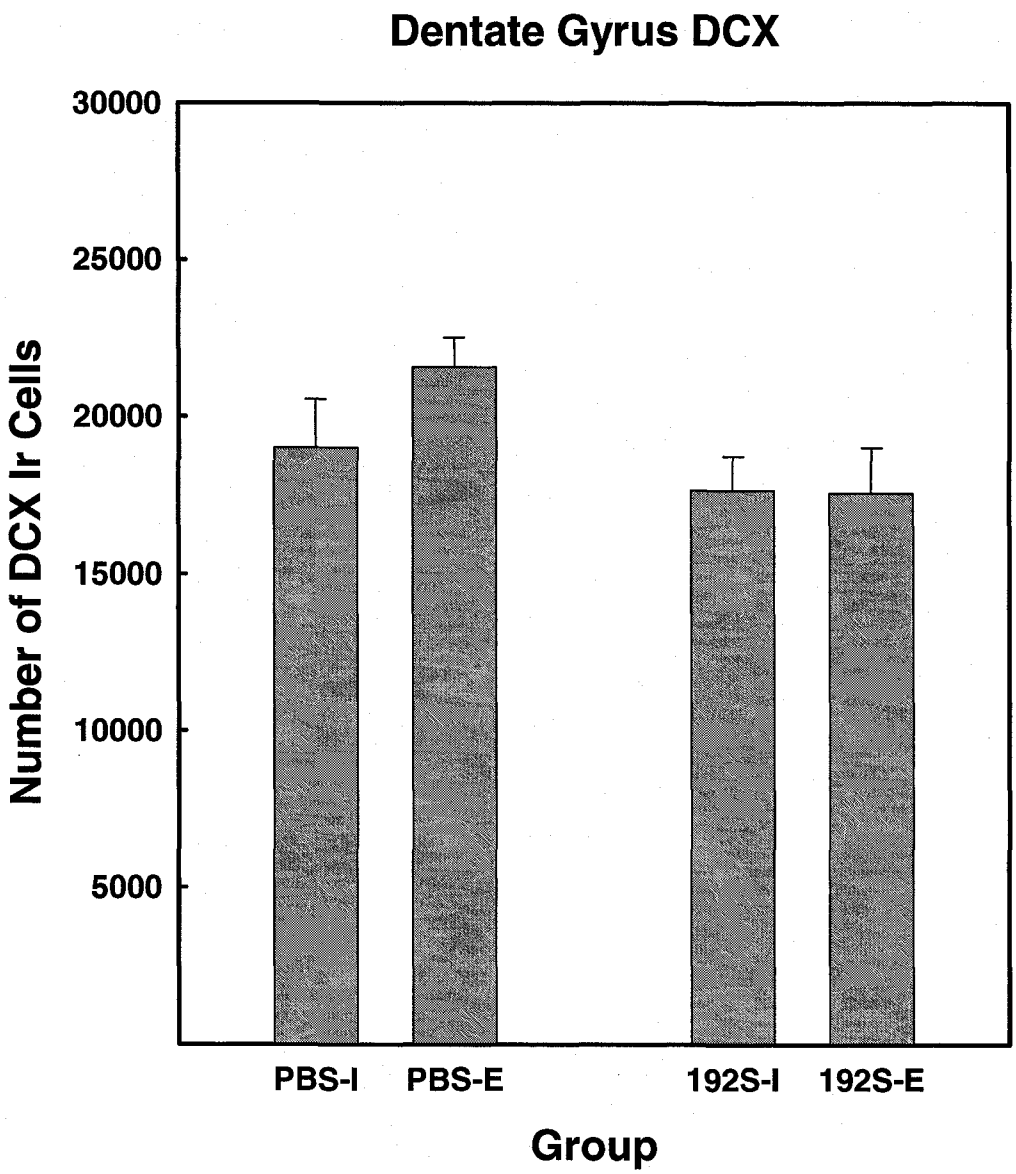
Separate ANOVAs were also performed to determine if there were effects of the 192S treatment for animals raised in either enriched or impoverished conditions. There was no significant lesion effect within either of these conditions for both the distance and the latency measure. However, the lesion effect on latency approached significance for both impoverished rats ($F(1,23)=3.75$, $p<0.065$) and enriched rats ($F(1,31)=3.68$, $P<0.064$).

Analysis of the swim speed data demonstrated a significant main effect for 192S ($F(1,54) = 4.49$ $p< 0.039$). 192S treated rats were slightly but significantly slower at swimming than the vehicle treated rats. No significant effect on speed was found for the housing condition. Post-hoc analysis showed that the lesioned rats swam significantly more slowly only on day 3 ($p<0.006$) and 4 ($p<0.023$).

Doublecortin (DCX) counts

Ten tissue sections per brain containing the hippocampus were stained for DCX for cohort 1 ($n = 39$). DCX immunoreactive (IR) cells were counted in the dentate gyrus using stereological techniques, resulting in unbiased estimates of total DCX IR cells per hippocampus. The results, shown in Figure 9, were analyzed by univariate ANOVA with between subjects factors being the housing condition and the lesion treatment. T-tests were then performed for comparisons between every group. The ANOVA of these dentate gyrus counts demonstrated only a significant main effect due to the lesion condition ($F(1,33) = 4.93$ $p<0.033$). The 192S treated animals had significantly lower

Figure 9. Estimated dentate gyrus DCX IR cell counts. The lesion groups are significantly different with respect to DCX IR counts ($F(1,33)=4.932$, $p<0.033$). The main effect for housing condition was not significant ($F(1,33)=2.047$, $p<0.162$). However, PBS-E group was significantly different from 192S-E ($t_{17}=2.43$, $p<0.03$) and 192S-I ($t_{17}=2.72$, $p<0.02$)



counts compared to the vehicle treated animals. The main effect for housing condition was not significant ($F(1,33)=2.047$, $p<0.162$). While there was a tendency for enrichment to increase the number of DCX IR cells in the PBS rats, this effect was not statistically significant ($t_{17} = 1.50$, ns). However, both the 192S-I (($t_{17} = 2.72$, $p< .02$) and the 192S-E ($t_{17} = 2.43$, $p< .03$) groups had significantly fewer DCX IR cells than the PBS-E group.

Pyramidal Cell Morphology

Quantitative assessment was performed on the pyramidal cell layer of the CA1 area of the hippocampal formation. A total of twenty Golgi-Cox stained brains were used for this analysis (five animals from each of the four groups). Ten pyramidal cells were quantified for each animal and the average of these ten cells for each variable comprised the scores for each animal. Figures 10-13 represent the tracing of one cell in each of the four groups. Figure 14 shows the summary data for each of the four groups for the number of dendritic branches, total branch length, total number of spines for the apical (left panel) and basal trees (right panel).

Analysis of the total number of branches, total branch length and number of spines was completed using univariate ANOVA with the 4 groups (192S-I, 192S-E, PBS-I and PBS-E) as independent variables.

Following that, repeated measures with the within subjects factor being the branch orders and the between-subjects factors being the housing condition and the lesion treatment, was used to look at the spine density, number of branches, total number of spines and total dendritic length all analyzed per branch order.

Figure 10. Sketch of representative pyramidal cell from the hippocampal CA1 region. Tracing of PBS-E (brain #8 section#4 cell#9) animal done with Neuroleucida software and analyzed through Neuroleucida explorer

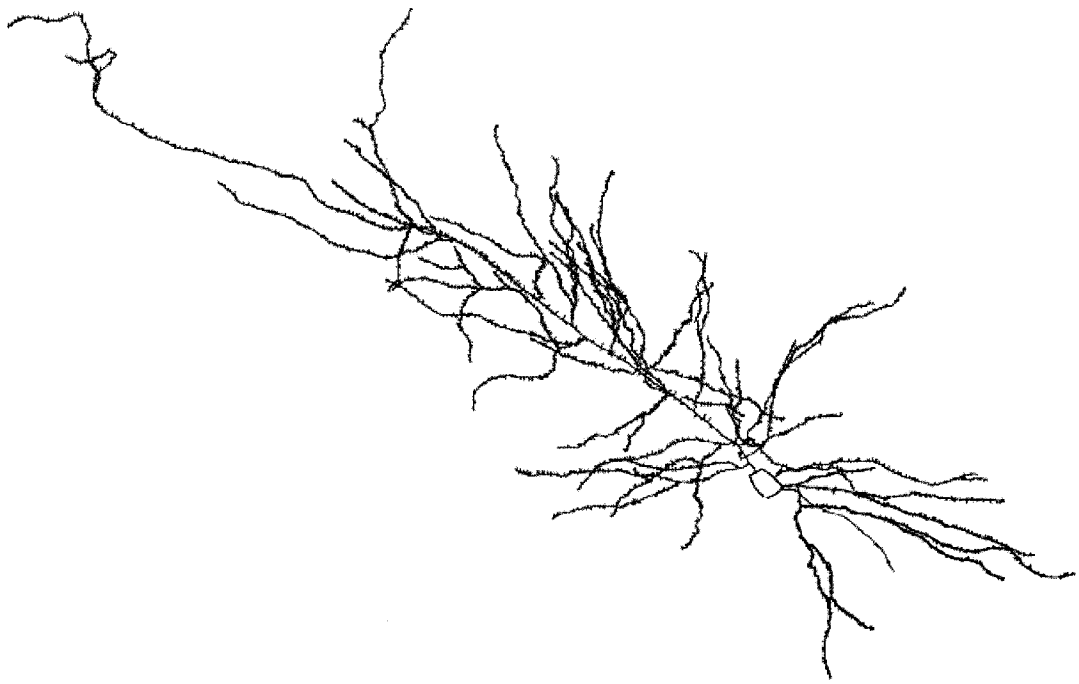


Figure 11. Sketch of representative pyramidal cell from the hippocampal CA1 region. Tracing of 192S-E (brain #16 section#4 cell#5) animal done with Neuroleucida software and analyzed through Neuroleucida explorer.

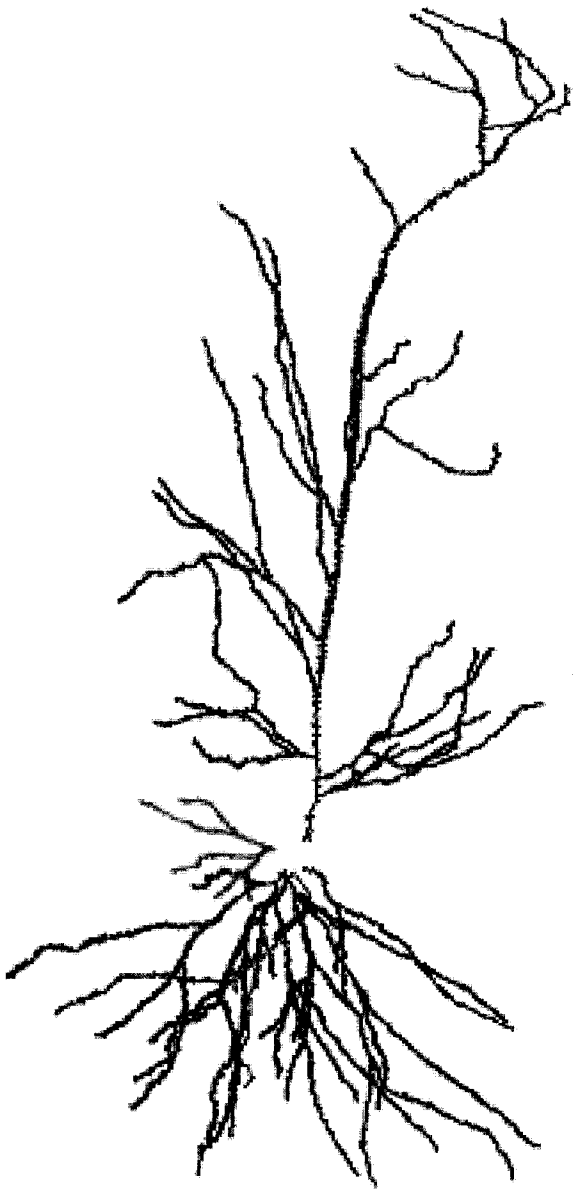


Figure 12. Sketch of representative pyramidal cell from the hippocampal CA1 region. Tracing of PBS-I (brain #27 section#5 cell#5) animal done with Neuroleucida software and analyzed through Neuroleucida explorer.

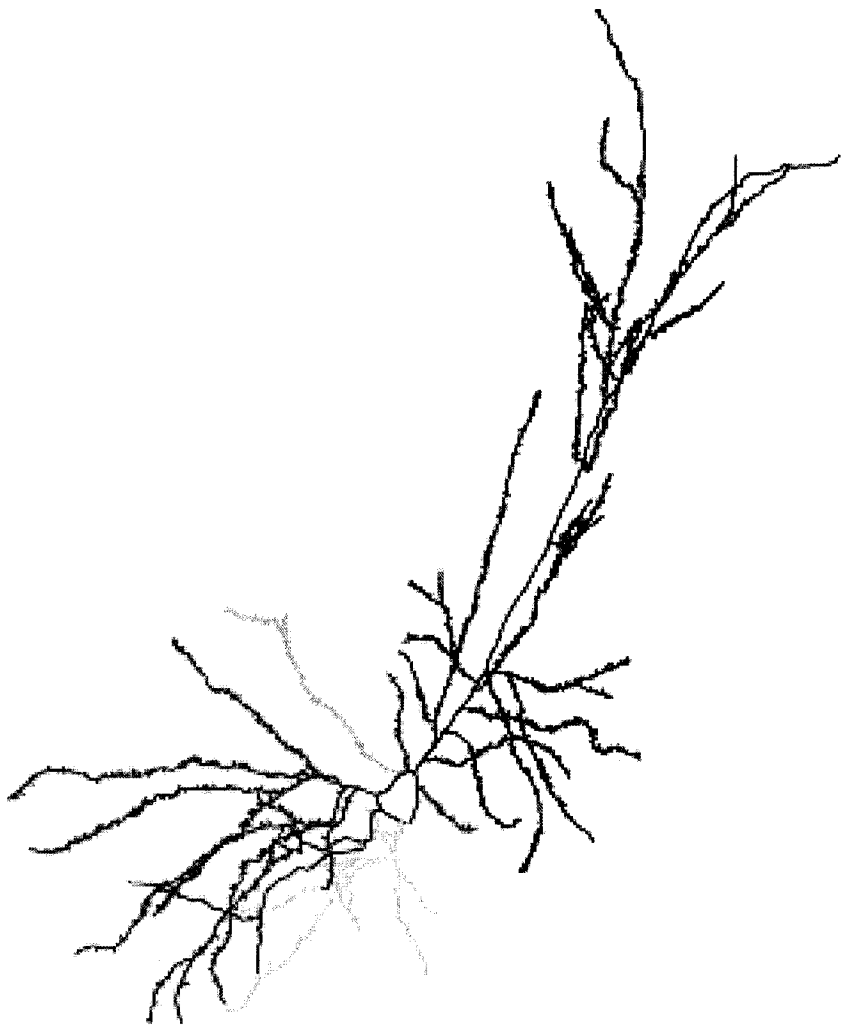
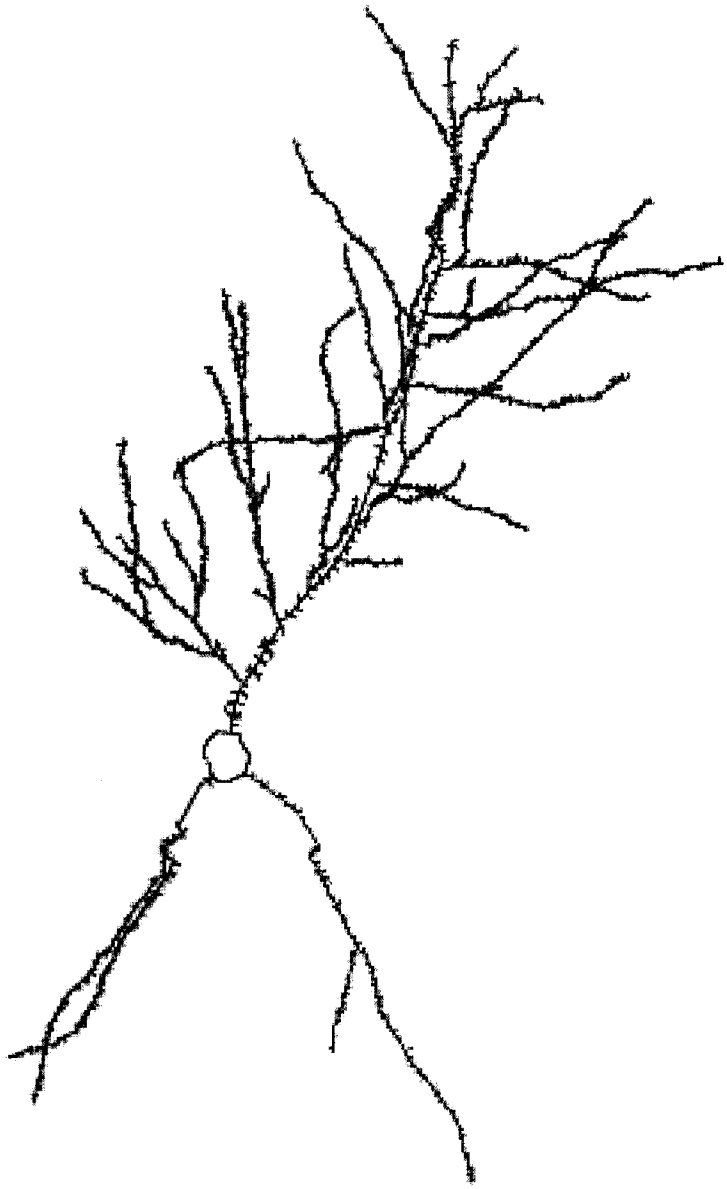


Figure 13. Sketch of representative pyramidal cell from the hippocampal CA1 region. Tracing of 192S-I (brain #37 section#5 cell#8) animal done with Neuroleucida software and analyzed through Neuroleucida explorer.



Total number of branches

The data for the basal and the apical tree (see Figure 14) were separately analyzed with univariate ANOVA for the total number of branches per cells. The 192S lesion effect was found to be significant in the apical tree ($F(1,16) = 7.57$, $p < 0.014$) but not for the basal tree ($F(1,16) = 0.81$, $p < 0.779$). The 192S lesioned animals had significantly less apical branches per cell than the non-lesioned animals. No significant effect was found for the housing condition in either the apical ($F(1,16) = 0.00$, $p < 0.985$) or basal ($F(1,16) = 0.04$, $p < 0.841$) trees.

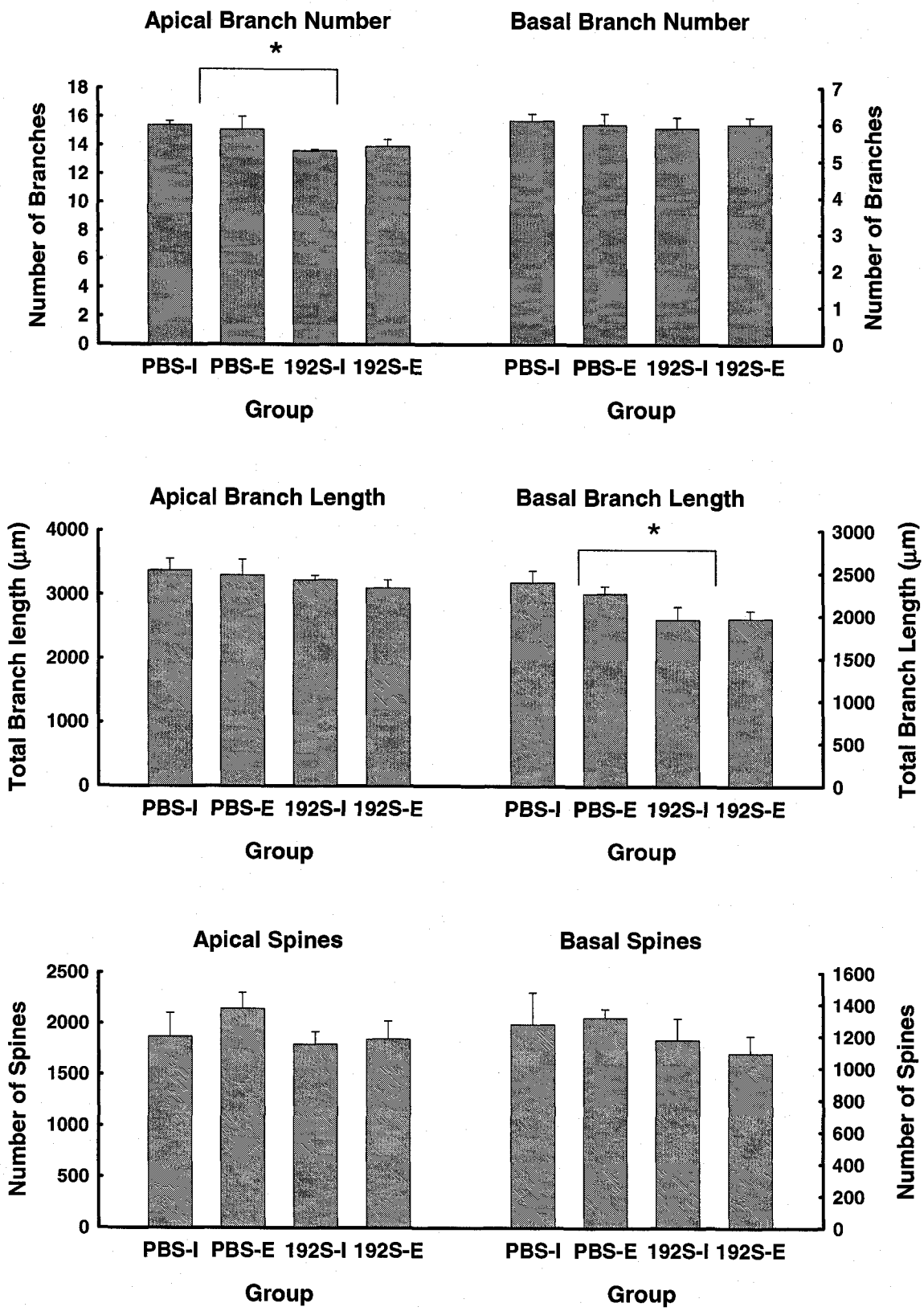
Total branch length

The total branch length (see Figure 14) per cell was analyzed with univariate ANOVA. No significant effect was found for the lesion condition in the apical tree ($F(1,16) = 1.10$, $p < 0.309$) but a significant effect was seen in the basal tree ($F(1,16) = 8.55$, $p < 0.010$). The lesioned animals showed significantly less basal branch length per cell than the non-lesioned animals. No significant effect was found for the housing condition on either the apical ($F(1,16) = 0.34$, $p < 0.56$) or basal ($F(1,16) = 0.22$, $p < 0.64$) trees.

Total number of spines

Univariate ANOVA was used to analyze the total number of spines for each group on both basal and apical tree (Figure 14). No significant 192S lesion effect was seen for either the apical ($F(1,16) = 1.08$, $p < 0.312$) or basal ($F(1,16) = 1.39$, $p < 0.255$) trees. Similarly, no significant effect was also seen in the housing condition on both apical ($F(1,16) = 0.80$, $p < 0.38$) and basal ($F(1,16) = 0.023$, $p < 0.88$) trees.

Figure 14. Shows the summary data for each of the four groups. Top panel represent the number of branches for the apical (left) and basal trees (right). Significant effect of the lesion found only on the apical tree ($F(1,16) = 7.57$, $p<0.014$). Middle panel represent the number of dendritic branches for the apical (left) and basal trees (right). Significant effect of the lesion found only on the basal tree ($F(1,16) = 8.55$, $p<0.010$). Bottom panel represent total branch length *(μm)* for the apical (left) and basal trees (right). No significant lesion effect was found for either apical or basal tree. No significant housing effect was found. * denotes significance.



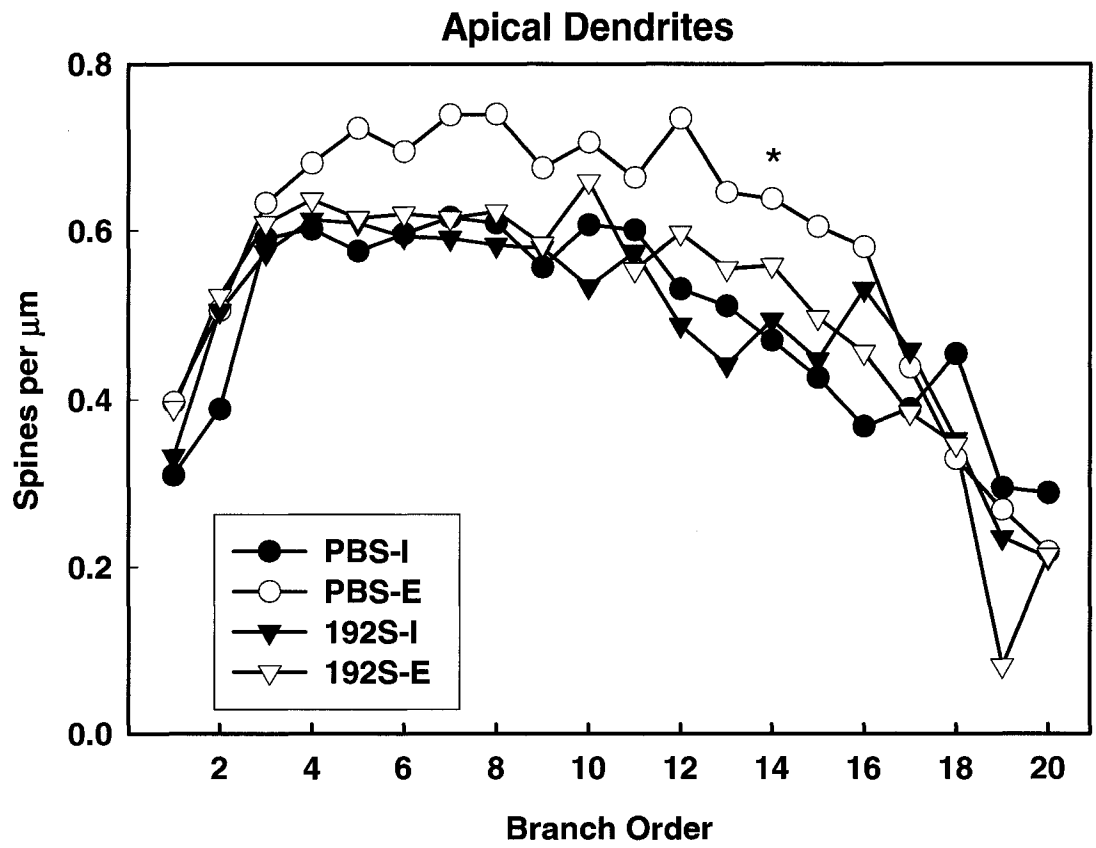
Spine density per Branch order

Repeated measures were used to analyzed the spine density. The within subjects factor was the first 20 branch orders and the between-subjects factors were the housing condition and the lesion treatment. One-way ANOVAs were also performed comparing the four groups at every branch order. There was no significant lesion effect on spine density (see Figure 15) across the first 20 branches for either the apical ($F(1,16) = 0.98$, $p< 0.33$) or basal ($F(1,16) = 0.03$, $p< 0.86$) trees. The housing condition also showed no significant effect on both apical ($F(1,16) = 2.917$, $p< 0.107$) and basal ($F(1,16) = 0.22$, $p< 0.645$) trees. However, when analyzed by one way ANOVA, the spine density was increased for apical branch order 14 with a significant enrichment effect in the PBS group ($F(1,8) = 8.90$, $p< 0.018$) and branch orders 1, 12, 15 and 16 just missed significance (all $p<0.1$). No significant effect was noticed in any of the other groups or in the basal tree analysis.

Number of branches per branch order

Repeated measures were used to compare all four groups. The number of branches per branch order (see Figure 16) were found not to differ between the lesion conditions for both apical ($F(1,16) = 0.10$, $p< 0.753$) and basal ($F(1,16) = 0.37$, $p< 0.551$) trees. There was also no significant effect reported for the housing condition on both the apical ($F(1,16) = 0.52$, $p< 0.480$) and basal ($F(1,16) = 0.81$, $p< 0.379$) trees. Separate ANOVAs demonstrated an increased number of branches in the enrichment animals for the apical PBS group at branch order 8 ($F(1,8) = 7.36$, $p< 0.027$). There was also an increased number of branches for the lesion animals on the apical tree for the impoverished group at branch order 5 ($F(1,8) = 6.06$, $p< 0.039$) and it just missed

Figure 15. Spine density analysis per branch order of the apical (upper panel) and basal (lower panel) trees. No overall significant effect of lesion and housing on both apical and basal tree. * denotes significant enrichment effect in 192S group, *denotes significant enrichment effect in PBS, * denotes significant effect of lesion in the I group and * denotes significant effect of lesion in the E group.



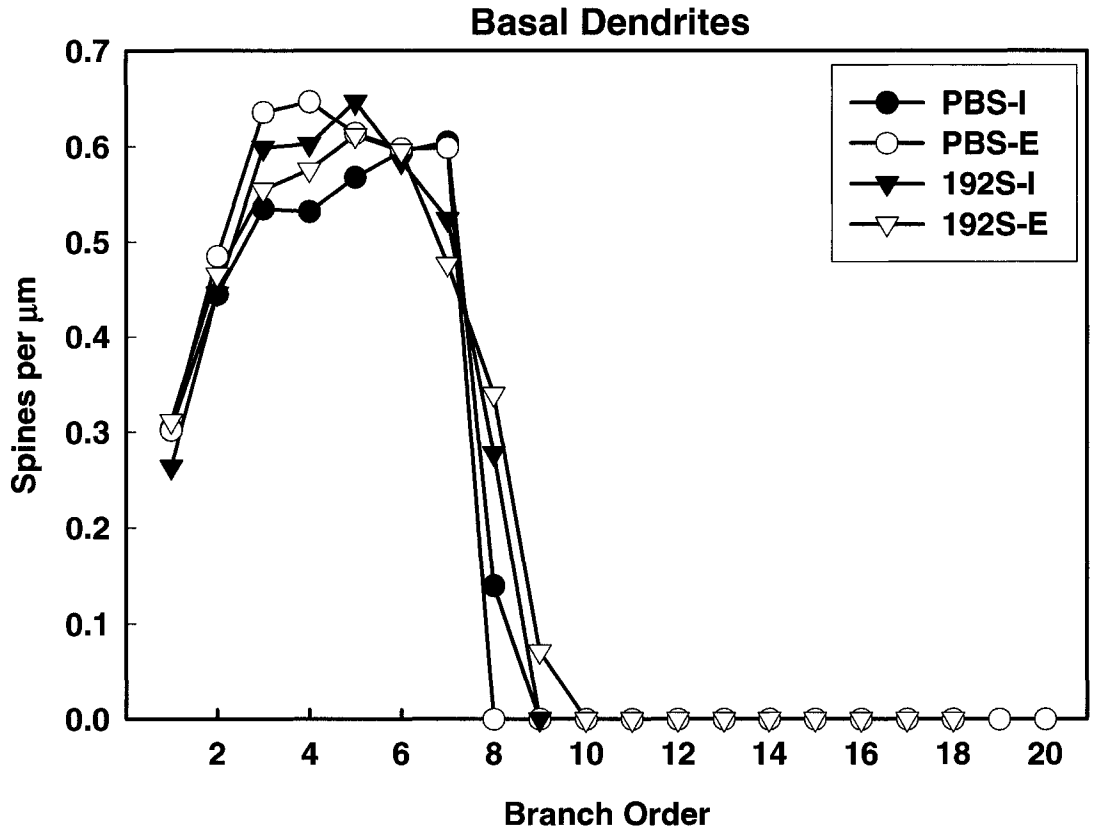
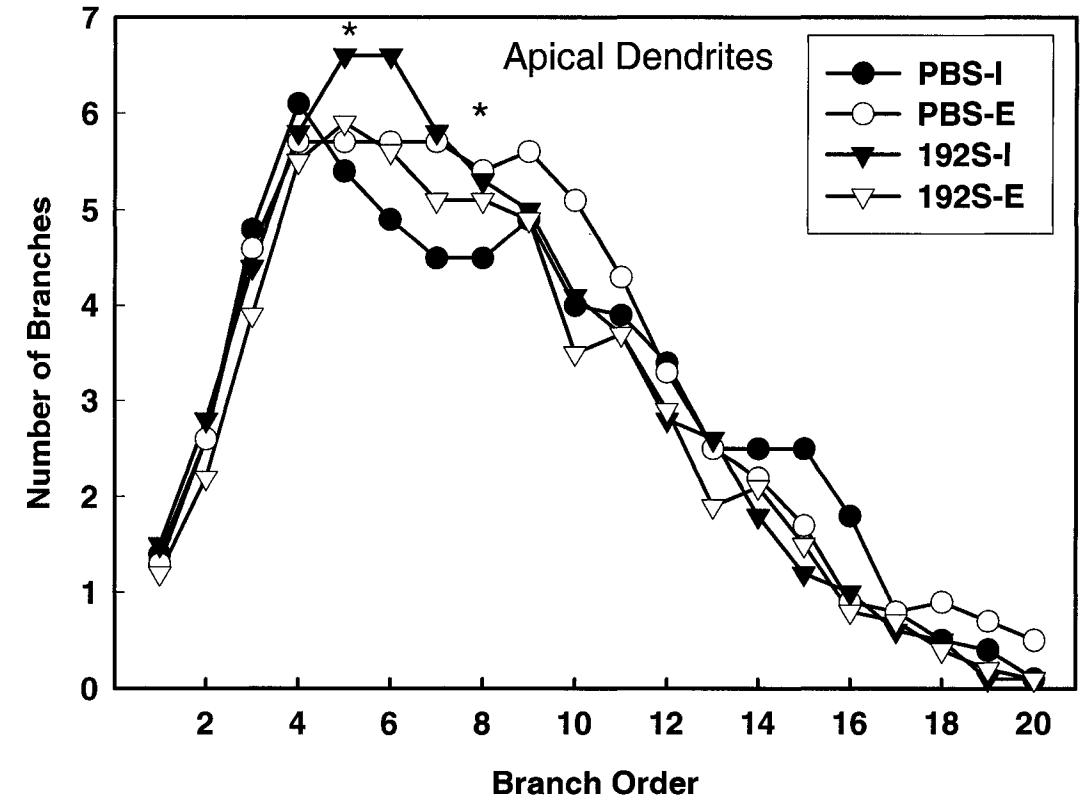
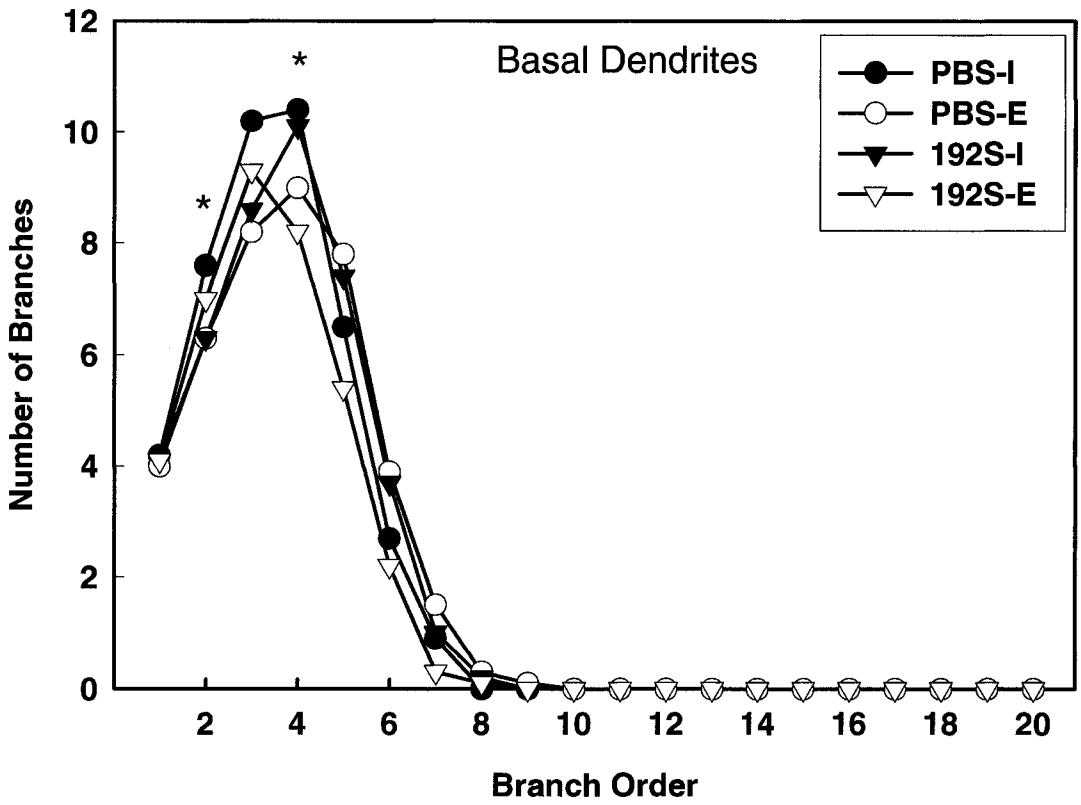


Figure 16. Number of branches analysis per branch order of the apical (upper panel) and basal (lower panel) trees. No overall significant effect of lesion and housing on both apical and basal tree. * denotes significant enrichment effect in 192S group, *denotes significant enrichment effect in PBS, * denotes significant effect of lesion in the I group and * denotes significant effect of lesion in the E group.





significance at branch order 8 ($p<0.1$). For the basal tree, there was a significant reduction effect of enrichment in the PBS group at branch order 4 ($F(1,8) = 5.52$, $p< 0.047$) and a lesion effect with reduced number of branches in the impoverished group at branch order 2 ($F(1,8) = 5.41$, $p< 0.049$).

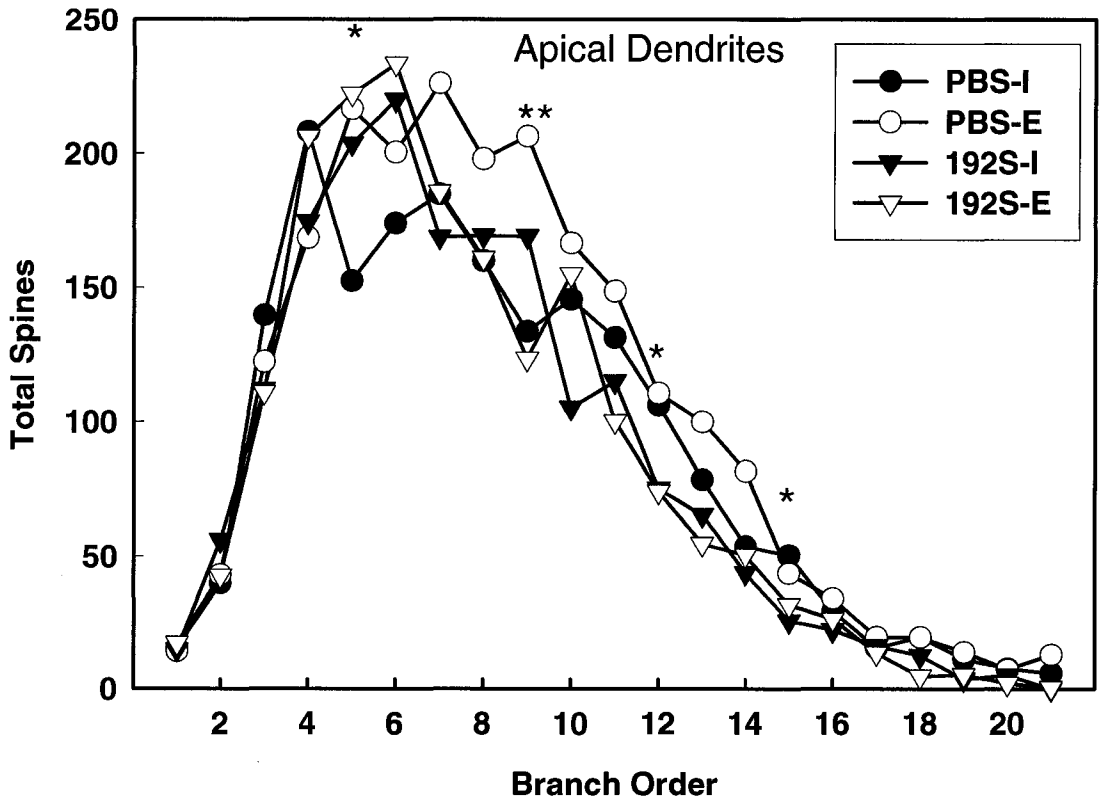
Total number of spines per branch order

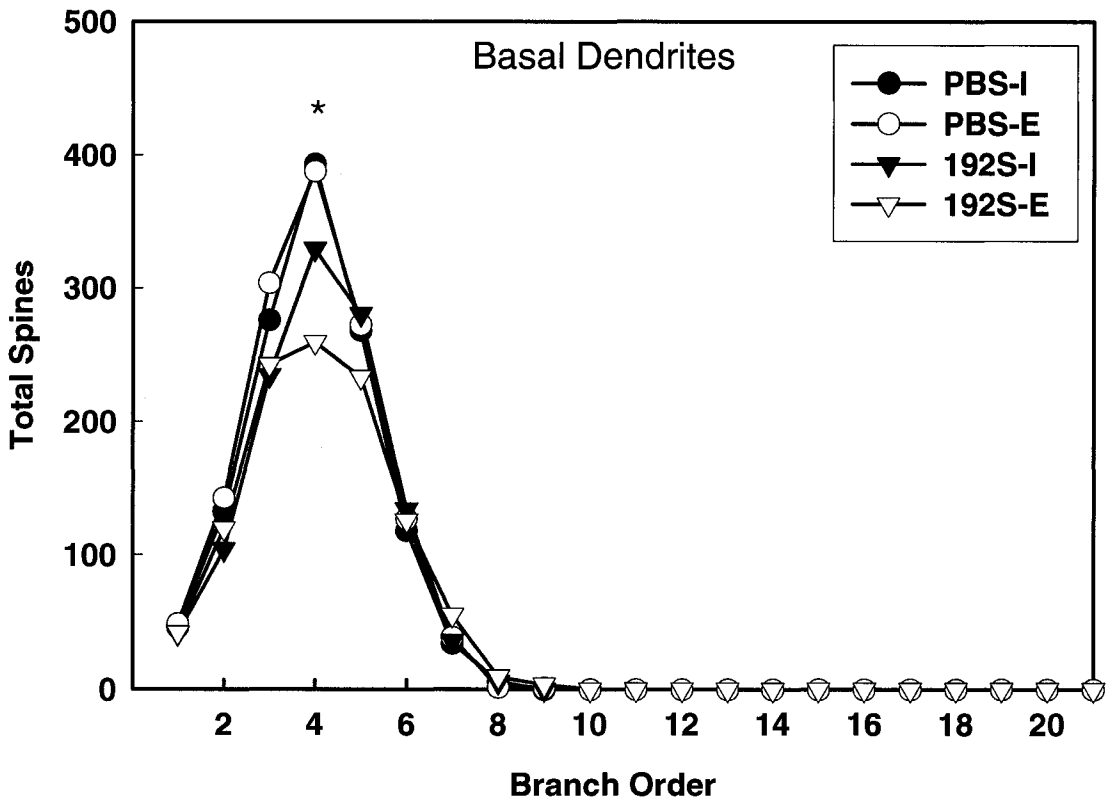
The total number of spines per branch order (shown in Figure 17) was analyzed with repeated measures. No significant effect was demonstrated by the repeated measures ANOVA for the lesion condition on the apical ($F(1,16) = 1.31$, $p< 0.26$) or basal ($F(1,16) = 1.53$, $p< 0.23$) trees and no significant effect was found for the housing condition on the apical ($F(1,16) = 0.89$, $p< 0.35$)and basal ($F(1,16) = 0.01$, $p< 0.93$) trees. Separate ANOVAs demonstrated an enrichment effect for increased number of spines in the apical PBS group at branch order 5 ($F(1,8) = 6.08$, $p< 0.039$) and branch order 9 ($F(1,8) = 13.55$, $p< 0.006$). There was also reduction in the number of spines demonstrating a lesion effect on the apical tree for the impoverished group at branch order 15 ($F(1,8) = 7.31$, $p< 0.020$). The enriched animals showed a lesion effect as lesioned animals showed reduced number of spines at branch order 9 ($F(1,8) = 18.61$, $p< 0.003$) and branch order 12 ($F(1,8) = 6.42$, $p< 0.035$) and this just missed significance at branch order 13 and 14 (all $p<0.1$). For the basal tree, a reduction in the number of spines demonstrated a significant lesion effect in the enriched group at branch order 4 ($F(1,8) = 12.01$, $p< 0.008$).

Total dendritic length (μm) per branch order

This analysis was performed with repeated measures on branch order. There was no significant effect resulting from the lesion treatment on the apical tree ($F(1,16) =$

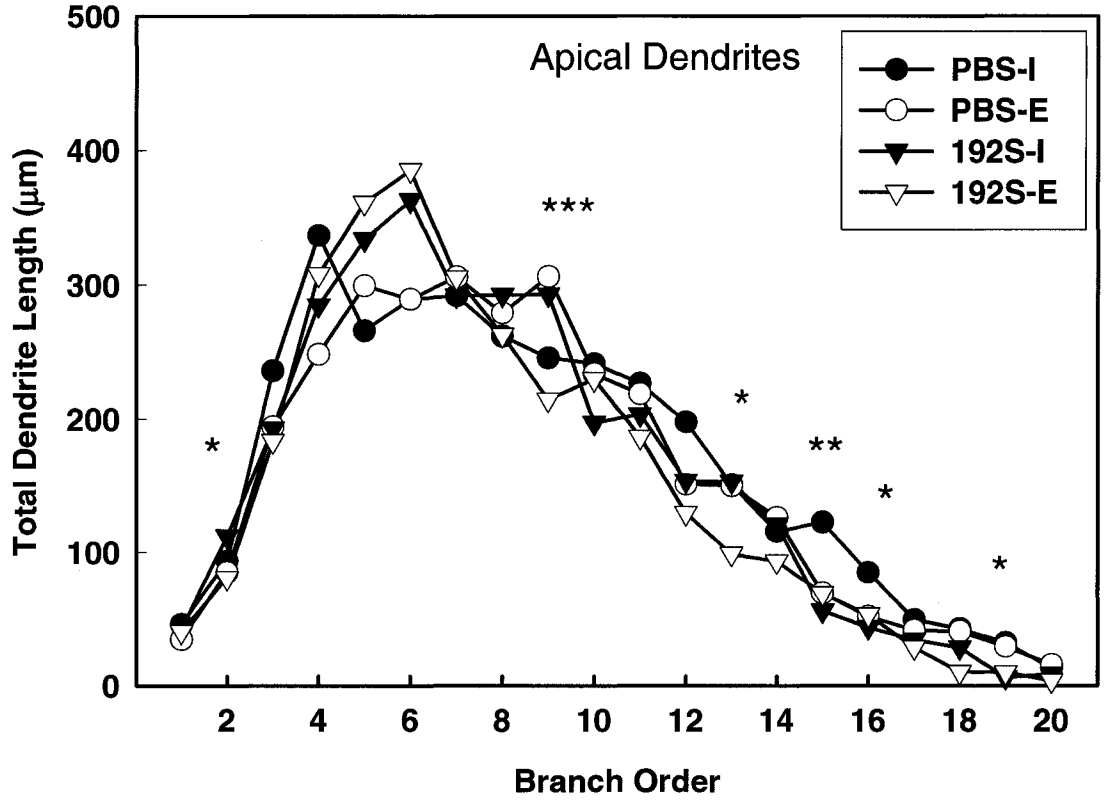
Figure 17. Total spines analysis per branch order of the apical (upper panel) and basal (lower panel) trees. No overall significant effect of lesion and housing on both apical and basal tree. * denotes significant enrichment effect in 192S group, *denotes significant enrichment effect in PBS, * denotes significant effect of lesion in the I group and * denotes significant effect of lesion in the E group.

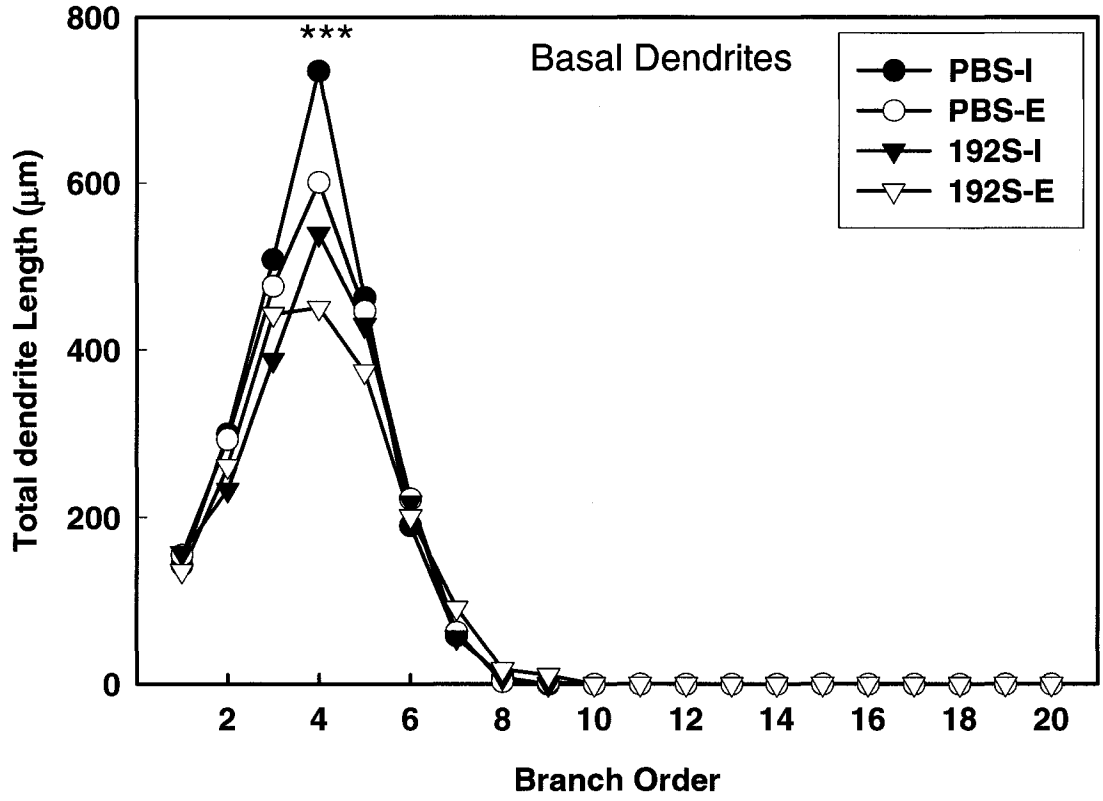




0.667, p< 0.426). However the basal tree showed significance for the lesion treatment ($F(1,16) = 6.492$, $p< 0.021$). No significant effect was found for the housing condition on both apical ($F(1,16) = 1.563$, $p< 0.229$) and basal ($F(1,16) = 0.534$, $p< 0.475$) trees. Separate ANOVAs demonstrated a reduced dendritic length for enrichment in the apical 192S group at branch order 2 ($F(1,8) = 13.62$, $p< 0.006$), branch order 9 ($F(1,8) = 6.10$, $p< 0.039$) and branch order 13 ($F(1,8) = 6.78$, $p< 0.031$) and branch order 18 just missed significance ($p<0.1$). There was also an increased dendritic length for the enrichment effect for the apical tree in the PBS group at branch order 9 ($F(1,8) = 6.15$, $p< 0.038$) and a reduction in dendritic length at branch order 15 ($F(1,8) = 5.56$, $p< 0.046$). There was a lesion effect on the apical tree for for reduced dendritic length in the impoverished group at branch order 15 ($F(1,8) = 20.84$, $p< 0.002$), branch order 16 ($F(1,8) = 8.74$, $p< 0.018$), branch order 19 ($F(1,8) = 7.51$, $p< 0.025$) and branch order 5 just missed significance ($p<0.1$). There was a lesion effect for reduced dendritic length in the apical tree of the enriched group at branch order 9 ($F(1,8) = 12.08$, $p< 0.008$) and branch order 13 just missed significance ($p<0.1$). The basal tree demonstrated significant enrichment effect for reduced dendritic length in PBS group at branch order 4 ($F(1,8) = 10.06$, $p< 0.013$). The lesion caused a reduction in dendritic length in the basal tree of the impoverished group at branch order 4 ($F(1,8) = 14.16$, $p< 0.006$) and branch order 2 and 3 just missed significance ($p<0.1$). The lesion also caused a reduction in dendritic length for the enriched group in the basal tree at branch order 4 ($F(1,8) = 21.61$, $p< 0.002$). The results are illustrated in Figure 18.

Figure 18. Total dendritic length analysis per branch order of the apical (upper panel) and basal (lower panel) trees. Significant lesion effect on the basal tree ($F(1,16) = 6.492$, $p < 0.021$). No overall significant effect of lesion on the apical tree and housing effect on both apical and basal tree. * denotes significant enrichment effect in 192S group, *denotes significant enrichment effect in PBS, * denotes significant effect of lesion in the I group and * denotes significant effect of lesion in the E group.





Discussion

The immunotoxin 192S was used to produce a forebrain cholinergic lesion in this study because of its high affinity for the p75 receptor, which is only present on cholinergic neurons of the basal forebrain (Yan and Johnson, 1988). Previous research showed that lesions produced with 192-IgG-saporin administration on postnatal day 7 resulted in a 80% loss of hippocampal choline acetyltransferase (ChAT) activity and a selective, long lasting loss of cholinergic neurons in the basal forebrain (Ricceri, Calamandrei and Berger-Sweeney, 1997; Ricceri et al., 1999; Pappas and Sherren, 2003; Pappas et al. 2005; Sherren and Pappas, 2005). Cholinergic lesions were performed here in 7-day-old male rats. Only males were used because sexual dimorphism has been reported in the development of the cholinergic system in rats (Ricceri, Calamendrei and Berger-Sweeney, 1997; Arter et al., 1998; Ricceri, Hohmann and Berger-Sweeney, 2002; Berger-Sweeney, 2003), although Sherren and Pappas (1999) did not observe any difference between male and female rats with respect to the extent of cholinergic lesion after the administration of 192S at 7 days of age. Nevertheless, only males were used in this study to prevent any potential added variability related to sex differences.

The decision to induce the cholinergic lesion 7 days postnatally was made because previous reports have demonstrated a more complete lesion of the system at this age compared to lesions performed either on postnatal day 1, 3 or 4 (Leanza et al., 1996; Ricceri, Calamandrei and Berger-Sweeney, 1997; Berger-Sweeney, 1998; Ricceri, Hohmann and Berger-Sweeney, 2002). This age (PND7) coincides with the peak in mRNA expression of $p75^{NTR}$, and therefore 192-IgG-SAP creates a more complete lesion of the system when injected then (Berger-Sweeney, 1998; Sherren, Pappas and Fortin,

1999). This time point also corresponds to the period when cholinergic basal forebrain fibers begin innervating the neocortex as well as the hippocampus (Berger-Sweeney, 1998).

As it turned out, the administration of 192S caused substantial and selective loss of cortical and hippocampal cholinergic innervation as shown by acetylcholinesterase and $p75^{NTR}$ IR. The lesions were graded in terms of the percentage of PBS control density of acetylcholinesterase staining and by semi-quantitative 4-point scale for $p75^{NTR}$ IR. These two measures were in complete agreement. Seven animals that exhibited only partial lesions based on these measures were excluded from the study. The most likely explanation is that there was a problem with the surgical procedure. As the pups were too small to use ear bars, the head of the animal could have been misplaced on the mould or may have moved slightly during the procedure, causing the injection to be made into the wrong location. There is always the possibility of a 192S batch effect (which we encountered in our pilot study) but this is less likely to be the case here since the animals that were discarded did not have surgery on the same day. Based on the severe reduction of both acetylcholinesterase staining and $p75^{NTR}$ IR, the lesions produced here are consistent with previous research showing a severe loss of choline acetyltransferase activity in the hippocampus (Pappas et al, 1996; Sherren and Pappas, 2005).

Behavioural testing: Morris Water Maze

Cholinergic lesioned animals were significantly impaired on the working memory version of the Morris water maze but only on the latency measure and not on the distance measure. These results indicate that the lesioned animals were slower at finding the platform compared to the non-lesioned animals, but still swam the same distance overall

before finding the platform. The swim speed of the lesioned animals was also found to be slightly but significantly slower than the non-lesioned rats, which in turn explains the significant finding for latency but not distance. We did not perform any test to assess the motor ability of the animals; however no obvious impairment was noticed during the water maze or at any other time. Neither motor impairment nor reduced swim speed has been reported previously by this and other laboratories conducting research on the effects of neonatal 192S, so the swim speed effect found here is anomalous if not spurious.

Since the lesioned and control rats did not differ on the distance variable, a measure of platform finding ability that is uncontaminated by swim speed, we conclude that the lesion did not affect the daily learning and memory of platform location in our working memory version of the Morris water maze. This is consistent with several other reports that showed no impairment of cholinergic lesioned rats on the reference memory version of the water maze, where the platform remains in the same location across test days (Leanza et al., 1996; Pappas et al, 1996; Sherren, Pappas and Fortin, 1999; Pappas et al., 2000). Indeed, impairment on the water maze task has been reported only for the first hidden platform training session (Sherren, Pappas and Fortin, 1999) or in aged, 22-months-old neonatal IgGS-lesioned rats (Pappas et al., 2005).

Pappas et al (2005) have proposed that neonatal cholinergic lesion has modest but discernible effects on problem solving ability whereas effects on attention and memory are not apparent. Hence, this lesion affects the animal's ability to learn difficult tasks such as the complex land (Pappas et al, 2000) and water based (Pappas et al, 2005) radial maze tasks. Ricceri (2003) has proposed that neonatal cholinergic lesions result in

learning deficits only on highly demanding tasks and tasks requiring long-term memory but not in tasks involving only short-term memory and a lower difficulty level.

Several studies show that cholinergic lesioning of the adult hippocampus also fails to affect spatial working memory (McMahan, Sobel and Baxter, 1997; Vuckovich, Semel and Baxter, 2007; Parent and Baxter, 2007). Thus, normal cholinergic innervation of the hippocampus is not essential for spatial learning/memory.

Environmental enrichment significantly enhanced the water maze performance of both the PBS control and 192S lesioned rats. The enriched animals performed significantly better on both distance and latency measures compared to rats housed in impoverished conditions. Early research showed that enrichment improves learning ability (Brown and King, 1971) and it was later demonstrated that major behavioural effects such as enhanced learning and spatial memory, increased exploratory activity and decreased anxiety can result from environmental enrichment (Nithianantharajah and Hannan 2006). The beneficial effect of enrichment is well known and widely reported on many different behavioural tasks including the Hebb-Williams maze, T-maze, open field and Morris water maze (Brown and King, 1971; Will and Rosenzweig, 1976; Kelche and Will, 1978; Ryan and Pappas, 1990; Kolb and Whishaw, 1998; Pappas et al, 1992; van Praag, Kempermann and Gage, 2000; Paban et al., 2005). Our results are consistent with recent literature reporting improved spatial learning/memory for otherwise normal animals raised in enriched environment (Pacteau et al., 1989; Wainwrigth et al., 1993; Nilsson et al., 1999; Gobbo and O'Mara, 2004; Hellemans, Benge and Olmstead, 2004; Will et al., 2004). Significantly, this facilitation was also observed here in animals that had sustained a neonatal forebrain cholinergic lesion.

One other study has looked at the combination of these two factors on memory and demonstrated that lesioned rats performed better on non-matching-to-position and object-recognition tests when housed in enriched environment post-lesion (Paban et al., 2005). In that study, the lesion was performed in 3-month-old rats instead of neonates. This outcome is consistent with the results of our experiment, showing the beneficial enrichment effect even when the housing condition and lesion are done later in the life of the animals.

Doublecortin (DCX) Counts

Neurogenesis in the dentate gyrus of neonatal cholinergic lesioned rats was investigated using DCX. DCX a is marker of newborn cells committed to a neuronal phenotype, in contrast to the commonly used marker bromodeoxyuridine (BrdU), which labels proliferating cells but not specifically neurons (Gould and Gross, 2002; Brown et al., 2003; Kempermann et al., 2004; Prickaerts et al., 2004; Rao and Shetty, 2004; Couillard-Despres et al., 2005; McDonald and Wojtowicz, 2005; Christie and Cameron, 2006; Koizumi et al., 2006; Plumpe et el. 2006). In this study we found that cholinergic lesioned animals exhibited significantly lower numbers of DCX positive cells in the dentate gyrus than the vehicle injected groups, indicating a decrease in neurogenesis. We have recently discovered a similar effect in 3 week old rat pups (Frechette and Pappas, unpublished) so the suppression of neurogenesis is an early appearing and long-lasting consequence of neonatal 192S lesion. This is the first study to examine the effects of neonatal cholinergic lesion on neurogenesis in the dentate gyrus, though a small number of studies have reported that adult forebrain cholinergic lesions impacts on neurogenesis in both rats and mice (Van der Borght et al., 2005; Cooper-Kuhn, Winkler and Kuhn,

2004; Mohapel et al., 2005). Mohapel et al., 2005 found that BrdU labelled DG cells were decreased after adult cholinergic lesion; however this study did not determine whether the decrease reflected a reduction in the production of neurons or other cell types (e.g. glia). Other researchers using double labelling for BrdU and NeuN (a marker for mature neurons) have reported that neuron production was decreased in cholinergically lesioned adult animals (Cooper-Kuhn, Winkler and Kuhn, 2004) and that the cholinergic system influences cells survival in hippocampal neurogenesis but not proliferation (Kaneko, Okano and Sawamoto, 2006; Kotani et al., 2006). Thus it seems that cholinergic denervation of the hippocampus, whether affected in infancy or adulthood, decreases neurogenesis in the dentate gyrus.

Overall, environmental enrichment did not significantly affect the number of doublecortin immunoreactive cells. However, there was a definite trend towards higher counts of newborn DCX-positive neurons in the PBS enrichment group compared to the PBS impoverished group. We believe that the effect would reach significance with a larger sample size since previous studies have shown that environmental enrichment increases neurogenesis in the dentate gyrus of the hippocampus in both rats and mice (Paylor et al., 1992; Kempermann et al., 1997a, b; Pham et al., 1999; van Pragg et al., 1999a, b; Brual-Juergerman et al., 2005; Olson et al., 2006). Interestingly, the DCX counts of the impoverished and enriched 192S groups were essentially identical with no hint of an enrichment effect. However, in the absence of a statistically significant effect of enrichment for the PBS rats, our enthusiasm to conclude that neonatal cholinergic lesion prevents the effects of enrichment on DG neurogenesis must necessarily be restrained. Clearly, future study of this interesting possibility is warranted.

A previous study demonstrated that depletion of serotonin in the hippocampus of adult rats subsequently exposed to enriched environment resulted in decreased BrdU labelled cells in the dentate gyrus (Ueda, Sakakibara and Yoshimoto, 2005). There was a significant reduction of newborn cells in the granule cell layer in lesioned animals as well as an increase in the number of cells in the control enriched group compared to the control standard housing group. Future studies should investigate the combination of neonatal cholinergic lesion and environmental enrichment with BrdU to determine how the proliferation versus survival of neurons is modulated by these combined factors. As well, the effects of cholinergic lesion should be compared with the effects of lesion to other selectively vulnerable neurotransmitter inputs to the hippocampus (e.g. serotonin, norepinephrine, dopamine). The effects of combined lesions also merit exploration.

Pyramidal Cell Morphology

The CA1 region of the hippocampus was chosen for morphological analysis of pyramidal cells because this region is heavily innervated by the basal forebrain cholinergic system and is sensitive to the effects of environmental enrichment (Walsh et al. 1995; Shepherd, 1998; van Praag, Kempermann and Gage, 2000; Lucas-Meunier et al., 2003; Paban et al., 2005; Nithianantharajah and Hannan 2006). Both cholinergic lesion and environmental enrichment have previously been shown to influence neural cytoarchitecture (Hohmann, Brooks and Coyle, 1988; West and Greenough, 1972; Greenough and Volkmar, 1973; Greenough, Volkmar and Juraska, 1973; Sherren and Pappas, 2005), and thus it was of interest to determine the combined effects of cholinergic lesion and environmental enrichment on CA1 pyramidal cell morphology.

Total number of branches and number of branches per branch order

The total number of branches was reduced by the lesion but only in the apical tree. No effect of enrichment was detected in either the apical or basal tree. No overall effect of the lesion or housing condition on the number of branches deriving from each of 20 branch orders on either the basal or apical trees was found by repeated measures ANOVA. However, separate analysis at each branch order showed an enrichment effect in the PBS group for the apical and basal tree. Enriched PBS animals showed higher number of branches. Previous research looking at the effects of environmental enrichment on CA1 pyramidal neurons has shown increased branches in both apical and basal trees due to enrichment (Faherty, Kerley and Smeyne, 2003). Here, we observed an enrichment effect in the PBS group but only at branch orders 4 and 8, and we are confident that a bigger effect would be observed with a larger sample size. Other reports show an increase in the number of branches in cortical pyramidal cells due to enrichment, either in the basal tree alone (Greenough, Volkmar and Juradka, 1973; Uylings et al., 1978) or in both basal and apical trees (Fiala, Joyce and Greenough, 1978; Wallace et al., 1992).

A lesion effect was found in the impoverished group for both the apical (branch order 5 was reduced in number) and basal tree (branch order 2 reduced in number). This reduction in the number of branches in the cholinergic lesioned animals is consistent with earlier reports demonstrating reduced branch number for pyramidal cells in the frontal cortex, retrosplenial cortex and visual cortex (Robertson et al., 1998; Works, Wilson and Wellman, 2004; Sherren and Pappas, 2005). However, this is the first report of this effect on CA1 pyramidal cells.

Total branch length and Total dendritic length per branch order

We found a significant reduction of total branch length for the lesion treatment, but only on the basal tree. No effect was detected for the lesion treatment on the apical tree, nor was any housing condition effect observed on either basal or apical trees. The same results emerged from the analysis of dendritic length per branch order where only the lesion effect was significant on the basal tree. While the overall ANOVA of all branch orders failed to show any significant effects, an enrichment effect was shown by the separate analysis of each branch order for the apical tree and was present in both the 192S and PBS group at specific branch order. Branch order 2, 9 and 13 showed significantly reduced branch length for the enriched group compared to impoverished animals whereas branch order 9 and 15 show significantly more branch length for the enriched group. There was also a lesion effect in both the impoverished and enriched group at branch orders 9, 15, 16 and 19 where the branch length was reduced for the lesioned animals. Analysis of the basal tree branches showed an enrichment effect in the PBS group at branch order 4 and a lesion effect in both the impoverished and enriched group. The significant overall reduction in total basal dendritic length as a result of the lesion is consistent with reports on cortical neurons (Robertson et al., 1998; Works, Wilson and Wellman, 2004; Sherren and Pappas, 2005). These studies report reductions in branch length for both the apical and basal trees of cortical pyramidal cells.

Previous research has indicated that dendritic length in CA1 pyramidal cells is increased as a result of environmental enrichment (Barthesaghi, Severi and Guidi, 2003; Faherty, Kerley and Smeyne, 2003). Similarly, total branch length and branch length per branch order of cortical pyramidal neurons are increased by enrichment (Uylings et al.,

1978; Green, Greenough and Schlumpf, 1983; Wallace et al., 1992). Here, enrichment effects were seen in the PBS and 192S groups at some branch orders only. A larger sample size would probably help reveal a stronger significant enrichment effect by reducing the variability within the groups.

Total number of spines, Spine density per branch order and Total number of spines per branch order

There was no significant overall effect for either the lesion or the housing condition on the total number of spines on the apical and basal trees. As well, the overall ANOVA of the number of spines per branch order demonstrated no significant effect of either the lesion or the housing condition on both basal and apical trees. However, ANOVAs of each branch showed an enrichment effect in the PBS group on the apical tree at branch orders 5, 9 and 14 for spine density and total number of spines, both of which were increased.

These observations on the lesioned animals do not concur with the only two other reports that have examined the effects of cholinergic lesion on dendritic spines, which report a decrease in the total number of spines or spine density in the visual cortex (Robertson et al., 1998), and medial prefrontal cortex (Sherren and Pappas, 2005). This is surprising in that neonatal 192S causes a greater degree of cholinergic deafferentation in the hippocampus than in the cortex (Pappas et al, 1996). However, this is the first study to examine hippocampal cytoarchitecture in cholinergic lesioned animals, and it is possible that the spine density of CA1 pyramidal cells is less responsive to cholinergic deafferentation than that of cortical neurons.

Previous research has indicated that enrichment normally increases the total number of spines as well as spine density in cortical pyramidal cells (Schapiro and Vukovich, 1970; Johansson and Belichenko, 2001; Kolb et al., 2003) and hippocampal pyramidal cells (Berman et al., 1996). In this study, enrichment increased spine density only at higher branch order. One possibility to explain the weak enrichment effect on spines in this study could be the age at which we killed the animals. The animals were only 60-65 days old, and are still considered to be young animals with developing brains. The total benefit from enrichment may not have been completed by the time we analysed their brains. Most enrichment studies discussed above analyzed the brains of animals that were 100 days of age or older (Fiala, Joyce and Greenough, 1978; Uylings et al., 1978; Green, Greenough and Schlumpe, 1983; Johansson and Belichenko, 2001; Berteshaghi, Severi and Guidi, 2003; Kolb et al., 2003).

Summary and conclusion

The objective of this project was to determine if neonatal cholinergic lesion and environmental enrichment would alter the behaviour of the animals on Morris water maze, the morphology of hippocampal pyramidal cells and the new born neurons in the dentate gyrus. We demonstrated in this study that the cholinergic lesion had no effect on Morris water maze performance. The environmental enriched animals on the other hand demonstrated better performance on the Morris water maze. Interestingly, this enrichment effect was observed in lesioned as well as non-lesioned animals.

The cholinergic lesion did impact on neurogenesis in the dentate gyrus, reducing the number of newborn neurons. Enrichment did not have a significant effect on neurogenesis although there was an apparent trend towards increased neurogenesis in the

non-lesioned animals. The mean number of newborn neurons in the enriched and impoverished lesion groups were identical and both means significantly differed from the enriched non-lesioned mean, suggesting no enrichment effect in the lesion group.

The results of the Golgi analysis were many and complex. They are summarized in Table 1. Clearly, the cholinergic lesion affected CA1 cell morphology, reducing apical branches and total basal branch length. This was not prevented by enrichment. There were also a number of other effects selective for certain branches but these effects tended to be observed equally often in impoverished and enriched rats. In other words, the effects of the cholinergic lesion were immune to the housing condition.

Enrichment had several effects but these less global but rather, selective for certain branch orders. Five of the six effects occurred only in the non-lesioned rats. The one effect that occurred in the lesioned rats was a reduction of the length of three apical branches (and the suggestion of a reduction in a fourth branch). The number of branches affected attests to the robustness of this effect yet its interpretation is obscure as enrichment would be expected if anything, to increase branch length. The most interesting consequence of enrichment, in terms of its implication for synapse density and information processing capability, is the increased spine density and spine number observed on some branches of the apical tree. This was evident only in the non-lesioned rats.

We conclude that neonatal cholinergic lesion has no effect on spatial learning/memory nor does it alter the facilitatory effects of enrichment on it. The lesion reduces neurogenesis in the dentate gyrus and may block the enhancing effect of enrichment on this neurogenesis. The lesion also alters the morphology of hippocampal

Table 1. Summary of the significant effects of forebrain cholinergic lesion and of enrichment on Golgi parameters analyzed. Bracketed items indicate effects that were close to significance ($p < 0.1$).

Cholinergic Lesion	Enrichment
Overall, reduced apical branches (not corrected by enrichment)	Increased spine density on apical tree at branch order 14 in PBS group ($p < 0.1$ for orders 1,12,15 and 16)
Overall, reduced basal branch length (not corrected by enrichment)	Increased number of branches on apical tree at branch order 8 in PBS group.
Increased number of branches on apical tree at branch order 5 in impoverished group ($p < 0.1$ for branch 8)	Reduced number of branches on basal tree at branch order 4 in PBS group
Reduced number of branches on basal tree at branch order 2 in impoverished group.	Increased number of spines on apical tree at branch order 5 and 9 in PBS group
Reduced number of spines on apical tree at branch order 15 in impoverished group	Reduced dendritic length on apical tree at branch order 2, 9 and 13 in the 192S group ($p < 0.1$ for order18)
Reduced number of spines on apical tree at branch order 9 and 12 in enriched group ($p< 0.1$ for orders 13 and 14)	Increased dendritic length on apical tree at branch order 9 in the PBS group
Reduced number of spines on basal tree at branch order 4 for enriched group	Reduced dendritic length on apical tree at branch order 15 in the PBS group
Overall, reduced basal dendritic length per branch order (not corrected by enrichment)	Reduced dendritic length on basal tree at branch order 4 in PBS group
Reduced dendritic length on apical tree at branch order 15, 16 and 19 in the impoverished group ($p < 0.1$ for order5)	
Reduced dendritic length on apical tree at branch order 9 in the enriched group ($p < 0.1$ for order 13)	
Reduced dendritic length on basal tree at branch order 4 in the impoverished and enriched group ($p < 0.1$ for orders 2 and 3)	

CA1 neurons, an effect which is immune to enrichment, and it seems to block the effects of enrichment with the greatest implication for synapse density and processing capacity, namely those on spine density and number.

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