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Date

LATERALIZATION OF THE DEVELOPING RAT HIPPOCAMPAL FORMATION

BY

AMANDA LYNN GROSS

B.A., Biology, Austin College, 2005

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Biology

The University of New Mexico
Albuquerque, New Mexico

December, 2011

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DEDICATION

I dedicate this dissertation to my mum, Linda Michael Anania. I thank her for teaching me to be brave, strong in will, and to fight for what I value. She made me believe that I could change the world; I am working on it, still. I am grateful for those parts of her that persist within me.

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LATERALIZATION OF THE DEVELOPING RAT HIPPOCAMPAL FORMATION

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ABSTRACT

Differences between the left and right hemisphere of the brain have been observed in humans and rodents (Broca, 1861; Wernicke, 1881), including the hippocampal formation, a region of the brain that is necessary for some forms of learning and memory (Olton and Samuelson, 1976; deToledo-Morrell et al., 1988; Bernasconi-Guastalla et al., 1994; Tabibnia et al., 1994; Poe et al., 2000; Lister et al., 2006; Hanlon et al., 2005; Sommer et al., 2005; Moskal et al., 2006; Thompson et al., 2008; Klur et al., 2009). Although lateralization of the hippocampal formation has been studied in the adult, few have sought to directly examine the development of hippocampal lateralization (Moskal et al., 2006) and none have examined hippocampal lateralization in the embryo.

The objective of the study outlined in this dissertation was to characterize the development of hippocampal lateralization in the rat. To achieve this objective, a rat CNS microarray with 1,178 genes representing the majority of ontological categories within the rat genome (Kroes et al., 2006) was used to examine lateralized gene expression in the embryonic rat hippocampal formation: 14 genes were all more highly expressed in the

right hippocampus at E18 (Gross et al., 2008; Gross et al., 2010). Database for Annotation Visualization and Integrated Discovery (DAVID) and Gene Set Enrichment Analysis (GSEA) were also used to further investigate whether specific genes differentially expressed at E18 comprised pathways known to be important in the development of the hippocampal formation. Results demonstrated that genes related to structure, transcription and translation, cellular metabolism, glycolysis, and gap junction signaling were more highly expressed in the right hippocampus at E18. Expression of genes corresponding to proteins that comprise the gap junction signaling pathway were further examined using qRT-PCR. Results showed that alpha1a-tubulin, beta3-tubulin, and connexin43 were more highly expressed in the right hippocampus at E18. Using Western blot analysis, alpha1a-tubulin protein levels were also shown to be higher in the right hippocampus at E18. These results indicated that genes related to hippocampal growth and development were more highly expressed in the right hippocampus at E18, and furthermore they suggested that gap junctions may play a critical role in the development of hippocampal lateralization in the embryo.

To further characterize the lateralized development of the rat hippocampal formation, the effect of N-methyl-D-aspartate glutamate receptor (NMDAR) mediated synaptic activity lateralized gene expression in the hippocampal formation during early postnatal development in the rat. During normal development, the pattern of lateralized gene expression displays a right-to-left shift in preferential expression between P6 and P9 (Moskal et al., 2006). A reduction in NMDA receptor (NMDAR) mediated synaptic activity using the selective NMDAR antagonist CPP, altered this pattern of lateralized gene expression at P9 (Rahimi et al., 2006, Gross et al., 2007; Claiborne et al., 2010).

These data were then analyzed using Significance Analysis for Microarrays, DAVID, and GSEA analyses. The MAPK signaling pathway was enriched in the right hippocampal formation at P9 following CPP injections: these data were corroborated and extended using qRT-PCR. Expression of MAPK14 mRNA was not significantly different between the left and right hippocampal formation at postnatal day 6, nor was it greater in the right HF as compared to the left in saline treated rats at P9; however, MAPK14 mRNA was more highly expressed in the right hippocampal formation at P9 following a reduction in NMDAR activity between P6 and P9. c-Myc was more highly expressed in the right hippocampal formation at P6, and it was not differentially expressed during normal development or following saline control injections between P6 and P9. However, cMyc mRNA expression was significantly greater in the right hippocampal formation in CPP treated rats. These findings indicated that genes involved in the MAPK signaling pathway were upregulated in the right hippocampal formation during early postnatal development following a reduction in NMDAR-mediated synaptic activity.

The findings presented in this dissertation are both novel and important: they are the first to demonstrate that lateralized gene expression is present in the embryonic rat hippocampal formation. Furthermore, these findings are the first to show the effect of early experience on the development of hippocampal lateralization in the first postnatal week. The results support the idea that differential gene expression patterns in the hippocampus are likely developmentally regulated and play a key role in the formation and function of that region and that the gene expression patterns can be significantly influenced by factors that modulate synapse plasticity.

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CHAPTER 1. INTRODUCTION

The study of lateralization is concerned with behavioral, anatomical, physiological neurochemical, and molecular differences between the left and right hemispheres of the brain, or a particular brain region. The first studies of lateralization focused on humans (Broca, 1861; Wernicke, 1881; Sperry 1961; Annett, 1964), as lateralization of the brain was once thought to be uniquely human (reviewed in Ghirlanda and Vallortigara 2004; Rogers et al. 2004; Vallortigara and Rogers, 2005). However, more recently, lateralization has been observed in other vertebrate species (Vallortigara et al., 1999; Ghirlanda and Vallortigara, 2004), including the rat (Myhrer and Iverson, 1990; Toga and Thompson, 2003; 2004; Cooke and Woolley 2005; Sun and Walsh, 2006; Ariffin et al., 2009). Importantly, lateralization has been shown to be functionally significant in both humans (Hanlon et al., 2005; Sommer et al., 2005; Erickson et al., 2007) and rodents (Cowell et al., 1997; Poe et al., 2000; Klur et al., 2009).

The objective of my dissertation was to characterize hippocampal lateralization during development. Many brain regions show differences between the left and right hemispheres, including the hippocampal formation, a region that is necessary for certain forms of learning and memory (Olton and Samuelson, 1976; deToledo-Morrell et al., 1988; Bernasconi-Guastalla et al., 1994; Tabibnia et al., 1994; Poe et al., 2000; Hanlon et al., 2005; Lister et al., 2006; Moskal et al., 2006; Thompson et al., 2008; Klur et al., 2009). Lateralization of the hippocampal formation has been observed in humans (Hanlon et al., 2005; Sommer et al., 2005; Thompson et al., 2008) and rodents (Bernasconi-Guastalla et al., 1994; Tabibnia et al., 1999; Poe et al., 2000; Tang et al., 2001; Verstynen et al., 2001; Kristofikova et al., 2004; Lister et al., 2006; Moskal et al.,

2006; Tang et al., 2008). Although lateralization has been shown to be functionally significant in adults, very little research has been directed toward the study of lateralization of the brain in development (Moskal et al., 2006; Sun et al., 2006; Thompson et al., 2008). Previously, Denenberg (Denenberg et al. 1981; Denenberg, 2005) argued that lateralization only emerges as a result of experience during postnatal development in the rat. In contrast, Sun and colleagues (2006) observed lateralized gene expression during embryonic development of the human cortex. These contrasting findings lead to two important questions related to the development of the brain: when is lateralization first established and how is it influenced by experience during early postnatal development?

To meet the objective of this dissertation, I sought to determine when lateralization of the hippocampal formation is first established by examining lateralization of gene expression in the rat hippocampal formation at E18. In order to study the embryonic development of hippocampal lateralization I examined asymmetric gene expression and protein levels. Our lab recently established that some genes were more highly expressed in the right hippocampal formation at postnatal day (P) 6 in the rat (Moskal et al., 2006); however, it had yet to be determined whether lateralization of gene expression can be observed at earlier points in hippocampal development. In addition to determining whether lateralized gene expression is observed during embryonic development, I also more specifically studied the pattern of lateralized gene expression in the embryonic rat hippocampus to identify molecular mechanisms that might underlie the establishment of hippocampal lateralization.

I further sought to determine whether lateralized gene expression at the end of the first postnatal week was influenced by a reduction in NMDAR-mediated synaptic activity. Available data suggest that hippocampal lateralization in the adult is influenced by NMDAR-mediated synaptic activity in development (Kristofikova et al., 2004) and that the NMDAR is differentially expressed in CA1 of the adult rat hippocampus (Kawakami et al., 2003; Wu et al., 2005; Shinohara et al., 2008); however, the more immediate effects of NMDAR-mediated synaptic activity on the development of hippocampal lateralization have yet to be determined. Our lab previously showed a right to left shift in lateralized gene expression between P6 and P9 in the rat hippocampal formation (Moskal et al., 2006), which coincides with changes in NMDAR-mediated synaptic activity that have an important role in the development of the hippocampal formation. For this reason, I chose to more closely examine the role of NMDA receptor mediated synaptic activity on the early postnatal development of hippocampal lateralization.

The NMDAR is thought to play a role in developing neural systems through the stabilization of excitatory synapses following receptor activation, and therefore previous investigators have used systemic injections of NMDAR antagonists to simulate the effects of a general reduction in sensory input during development (Sanes et al., 2006; Constantine-Paton, 1994). The NMDAR has an important role in the development of the hippocampal formation during early postnatal development. NMDAR density has been shown to increase in the hippocampal formation over the first postnatal week (Tremblay et al., 1988; Bellone and Nicoll, 2007). Importantly, the NMDAR has also been shown to modulate neuronal development of the hippocampal formation during the first postnatal week by affecting the maturation of neurons (Sanchez et al., 2001; Jones et al., 2003) and

has been proposed to be important in the formation of normal neural networks through appropriate synapse formation (Constantine-Paton, 1994; Battistin and Cherubini, 1994; Ben-Ari et al., 1997; Frotscher et al., 2000; Luthi et al., 2001; Bellone and Nicoll, 2007). Furthermore, our lab showed that the intraperitoneal injection of the NMDAR antagonist 3-((+/-)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP; Harris et al., 1986) blocked long-term potentiation (LTP) induction in dentate granule cells following stimulation of the medial perforant path axons at the end of the first postnatal week *in vivo*, indicating that NMDAR activity is important at this stage in hippocampal development (O'Boyle et al., 2004).

In the following dissertation, I first described the development of the hippocampal formation in Chapter 2 to provide a basic framework for understanding the implications of when lateralized gene expression is first established in the rat hippocampal formation and how the specific pattern of gene expression observed during embryonic development and at the end of the first postnatal week might affect hippocampal development. In Chapter 3, I describe the study of lateralization in humans and rodents. Furthermore, in Chapter 4, I argue why studying lateralization of the hippocampal formation specifically is of interest to the study of hippocampal development and the broader implications hippocampal asymmetry has in the study of adults. In Chapter 5, I sought to determine when lateralized gene expression is first established and in Chapter 6, I focused on the influence of experience and a reduction in NMDAR-mediated synaptic activity on lateralized gene expression at the end of the first postnatal week. Lastly, in Chapter 7, I summarize the results from Chapters 5 and 6 and argue that the patterns of lateralized gene expression observed at specific points in development and following specific

experimental manipulations are likely to influence the development and function of the hippocampal formation.

CHAPTER 2. THE RAT HIPPOCAMPAL FORMATION

The goal of this chapter is to discuss the embryonic and early postnatal development of the rat hippocampal formation. Prior to describing the development of the hippocampal formation, I will first include a brief discussion of the adult rat hippocampal formation in order to place the timeline of embryonic and early postnatal hippocampal development in the context of adult structure and function. I will then include a review of the development of the hippocampal formation. This review will provide context for understanding left-right differences observed during hippocampal development and in the adult rat, which is discussed in detail in Chapter 4.

Anatomy of the Adult Rat Hippocampal Formation

The hippocampal formation is comprised of the hippocampus proper (CA1, CA2, and CA3; Lorente de No, 1934) and the dentate gyrus (Cowan, 1980), although, in some cases the term "hippocampal formation" is also used to refer to a region that also includes the subiculum, presubiculum, parasubiculum, and the entorhinal cortex (Amaral and Witter, 1995; Johnston and Amaral, 2004; Amaral and Lavenex, 2007). CA3 and CA2 are sometimes called *regio inferior* and CA1 is also referred to as *regio superior* (Cajal 1893; 1901). For the purposes of this dissertation, the term hippocampal formation will refer only to the hippocampus proper and the dentate gyrus, and the term hippocampus will refer only to the hippocampus proper (Figure 2.1).

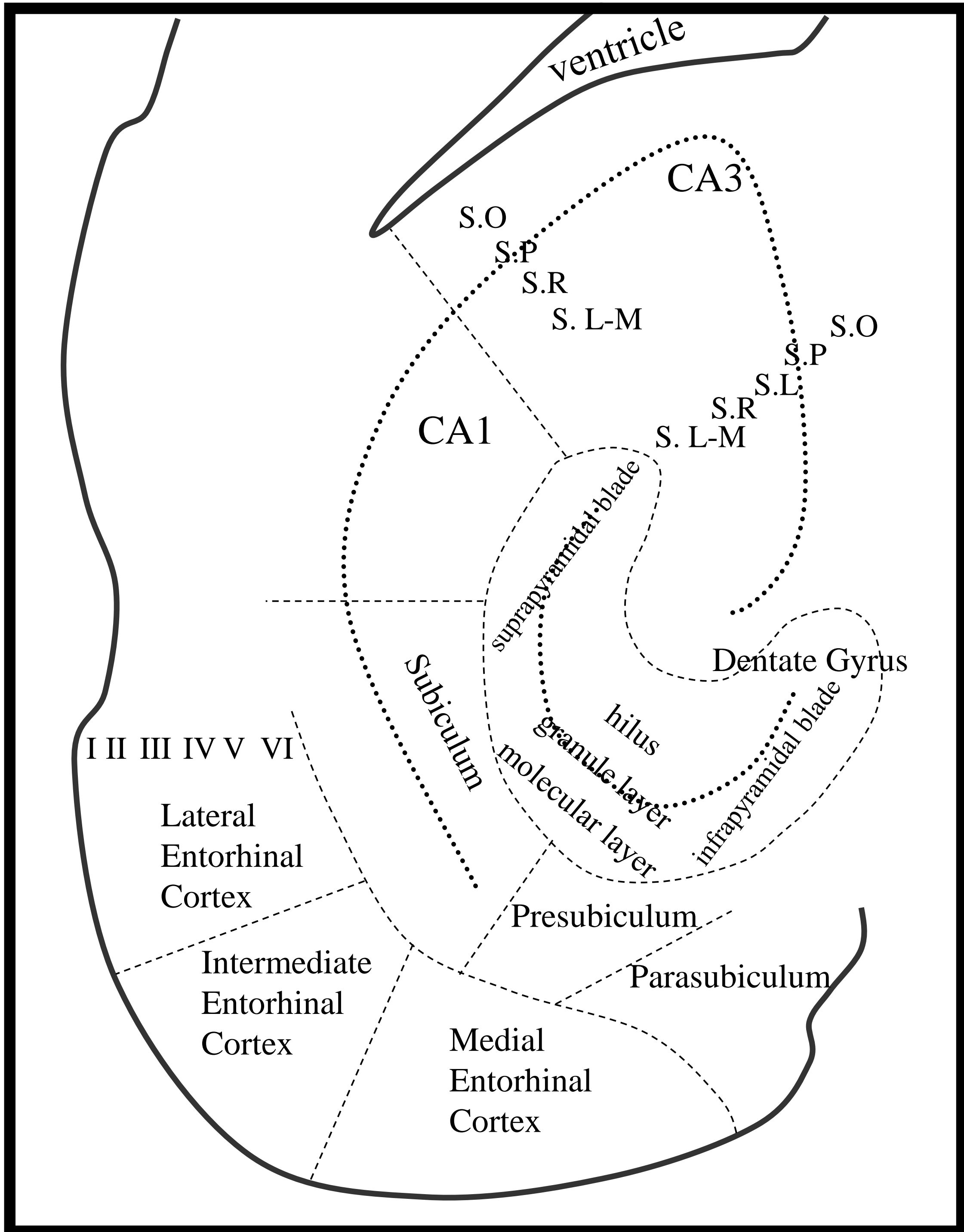

Figure 2.1: *Horizontal view of the hippocampal region.* Subregions of the hippocampal region are separated by dashed lines and the pyramidal and granule cell layer of the hippocampal formation are defined by dotted lines. List of abbreviations: S.O: stratum oriens, S.P: stratum pyramidale, S.L.: stratum lucidum, S.R: stratum radiatum, S. L-M: stratum lacunosum-moleculare.

In the past, the hippocampal formation was often discussed as a trisynaptic circuit (Andersen et al., 1971); however, the synaptic connections between subregions of the hippocampal formation and the hippocampal region are far more complex (Freund and Buzsaki, 1996; Johnston and Amaral, 2004; Amaral and Lavenex, 2007). Here I will first define each of the subregions of the hippocampal formation individually, including the principal cells and interneurons found within those subregions. Second, I will describe the extrinsic afferent input to the hippocampal formation from other brain regions. Third, I will delineate the intrinsic connections between subregions of the hippocampal formation. Lastly, I will discuss the efferent axonal projections from the hippocampal formation to other regions of the brain.

Dentate Gyrus

The dentate gyrus is a trilaminate cortical region divided into the stratum moleculare, stratum granulosum, and the hilus (reviewed in Amaral and Lavenex, 2007; Rahimi and Claiborne, 2007; Figure 2.1). The stratum moleculare, or molecular layer, is further subdivided into the outer, middle, and inner molecular layers (Bayer, 1980a; 1980b) that in total average about 3,221 μm (range of 2,324 – 4,582μm) in thickness in the adult rat (Claiborne et al., 1990). The stratum granulosum, or granule cell layer, is where the cell bodies of dentate granule neurons are located and is 40-80 μm in thickness in an adult rat (Claiborne et al., 1990). The hilus is located between the upper (suprapyramidal) and lower (infrapyramidal) blades of the dentate gyrus and is bordered by the CA3c region of the hippocampus proper (reviewed in Amaral and Lavenex, 2007; Figure 2.1).

Granule cells are the principal cells of the dentate gyrus and number approximately 1.2 million in the adult rat (Boss et al., 1985; West et al., 1991; Witter, 1993; Rapp and Gallagher, 1996). The cell bodies of dentate granule neurons in the adult rat are about 10 µm in diameter and typically have between one and four main apical dendrites that branch, project into the molecular layer, and extend to the hippocampal fissure. The transverse spread of these dendrites is on average 325 µm and the septotemporal spread averages 176 µm (Claiborne et al., 1990). Adult granule neurons located in either the suprapyramidal or infrapyramidal blade display differences in dendritic architecture. Suprapyramidal cell dendrites have significantly greater average total lengths (3500 vs. 2800 µm), transverse spreads (347 vs. 288 µm), number of segments (31 vs. 27), and number of spines (1.6 vs. 1.3 spines/µm) as compared to dendrites of the infrapyramidal blade (Desmond and Levy, 1985; Claiborne et al., 1990). The axons of the granule cells are called mossy fibers and originate from the basal portion of the granule cell body.

The principal cell of the hilus is the mossy cell (Amaral, 1978). The cell bodies of mossy cells in the rat are 25-35 µm in diameter and have three or more dendrites that project for long distances throughout the hilus (Buckmaster et al., 1992, 1996). Proximal dendrites of mossy cells have dense large complex spines called thorny excrescences (much denser than those located in stratum-lucidum of CA3). Mossy cells are glutamatergic cells and have axons that project to the inner molecular layer of the ipsilateral and contralateral dentate gyrus (Buckmaster et al., 1996; reviewed in Amaral and Levenex, 2007).

In addition to the principal cells of the dentate gyrus, there are also numerous other types of neurons, most of which are inhibitory interneurons. Interneurons of the dentate

gyrus include the chandelier, basket, molecular layer perforant path associated cell (MOPP), hilar commissural-associational pathway-related (HICAP) cell, and the hilar perforant path-associated cell (HIPP) cell (reviewed in Freund and Buzsaki, 1996; reviewed in Amaral and Lavenex, 2007). In the rat, the cell bodies and dendrites of chandelier cells are located within the molecular layer and the axon descends from the molecular layer into the granule cell layer to synapse on the initial axon segments of as many as 1,000 dentate granule neurons (reviewed in Freund and Buzsaki, 1996). The basket cell bodies are 25 to 35 μm in diameter and are located within the granule cell layer (reviewed in Freund and Buzsaki, 1996). These cells have aspiny apical dendrites that extend into the molecular layer and several basal dendrites that extend into the hilus (Ramon y Cajal, 1901). The axons of basket cells synapse on the cell bodies of nearby dentate granule neurons within the granule cell layer to inhibit those cells (Seress and Pokorny 1981; reviewed in Freund and Buzsaki, 1996). MOPP cell bodies are located within the molecular layer and have aspiny dendrites that remain within the layer and an axon that synapses on granule cell dendrites within the outer and middle molecular layers (Halasy and Somogyi 1993a, 1993b; reviewed in Freund and Buzsaki, 1996).

The HICAP and HIP cells are interneurons located specifically within the hilus of the dentate gyrus (reviewed in Freund and Buzsaki, 1996; Amaral and Lavenex, 2007). HICAP cells have aspiny dendrites that extend within the hilus and the axon of these cells project to the inner molecular layer of the dentate gyrus and act to inhibit granule cell activity. The HIPP cell body is located within the hilus and has two to three principal dendrites that remain within the hilus and often have long, branched spines. The axons of

HIPP cells extend into the outer and middle molecular layers with as many as 100,000 synaptic terminals that inhibit granule cell activity (Amaral, 1978).

Hippocampus Proper

The hippocampus proper is commonly divided into cornu ammonis regions (typically CA1, CA2, and CA3; Lorente de No, 1934). These subregions of the hippocampus proper are defined based on two criteria: first, the size of the pyramidal cell bodies, and second, the presence or absence of mossy fiber synaptic input from dentate granule neurons. Pyramidal cells of CA2 and CA3 have larger cell bodies as compared to those in CA1, but only pyramidal cells of CA3 receive afferent input from mossy fiber axons (Lorente de No, 1934). CA3 is the subregion of the hippocampus proper located closest to the dentate gyrus. Lorente de No (1934) further divided CA3 into CA3c (that is closest to the hilus between the suprapyramidal and infrapyramidal blade), CA3b, and CA3a (the curved segment of CA3).

The principal cell layer of the hippocampus proper is composed of pyramidal cell bodies. The layer located deep to the pyramidal cell layer is the stratum oriens, which contains interneurons and the basal dendrites of pyramidal cells. In the CA3 field, and importantly not in CA2 or CA1, the stratum lucidum layer is immediately above the pyramidal cell layer and dendrites of CA3 pyramidal cells located within stratum lucidum receive mossy fiber axon input. Stratum radiatum is superficial to stratum lucidum in CA3; in contrast, stratum radiatum is located directly above the pyramidal cell layer in CA2 and CA1. In all CA regions, the stratum lacunosum-moleculare is the most superficial layer. Strata lucidum, radiatum, and lacunosum-moleculare contain the apical

dendrites of pyramidal cells and various interneurons (reviewed in Amaral and Lavenex, 2007; Figure 2.1).

The principal cells of the hippocampus proper are pyramidal cells (Ishizuka et al., 1995). In the rat, CA3 pyramidal cell bodies (20-30 µm in diameter) are located in stratum pyramidale (60-120 µm in length) with one or two prominent apical dendrites that extend into strata lucidum (width of 471 µm), radiatum (width of 4,382 µm), and lacunosum-moleculare (width of 1,983 µm) and basal dendrites that extend into stratum oriens (width of 5,645 µm, Ishizuka et al., 1995). The proximal apical dendrites have large complex spines, called thorny excrescences (about 41 per neuron in the rat) that are located primarily within stratum lucidum, with a few in stratum oriens, and they specifically receive afferent input from mossy fiber axons (Ishizuka et al., 1995; Gonzales et al., 2001). The total dendritic lengths of CA3 pyramidal neurons range from 9,300 µm in CA3c to 15,800 µm in CA3a, and average 12,482 µm for the entire CA3 region in the rat (Ishizuka et al., 1995).

Pyramidal cells of CA2 most closely resemble those of CA3a; however, they lack thorny excrescences (Ishizuka et al., 1990; Ishizuka et al., 1995). In the rat, CA2 pyramidal cells have less total dendritic length in stratum oriens (5,865 µm) and greater dendritic length in stratum radiatum (4,798 µm) and lacunosum moleculare (4,671 µm) as compared to cells of CA3a. The total dendritic length of a CA2 pyramidal cell averages 15,405 µm (Ishizuka et al., 1995).

In the rat, pyramidal cell bodies of CA1 (15 µm in diameter) are located in stratum pyramidale with one or two apical dendrites that extend into strata radiatum (6,306 µm) and lacunosum-moleculare (2,531 µm), and basal dendrites that extend into stratum

oriens (4,586 μm). CA1 pyramidal cells with a single apical dendrite have larger basal dendritic trees, whereas, CA1 pyramidal cells with two apical dendrites have greater total apical dendritic length. In some instances a CA1 neuron with a single apical dendrite will branch within stratum radiatum or lacunosum-moleculare: those that branch are considered bifurcating CA1 neurons, while those that do not are considered non-bifurcating. The total length of the dendritic tree of a CA1 pyramidal neuron in the rat averages 13,424 μm (Ishizuka et al., 1995).

Interneurons of the hippocampus proper include the chandelier (axo-axonic) cell, basket cell, bistratified cell, horizontal trilaminar cell, radial trilaminar cell, the oriens lacunosum-moleculare (OLM) cell, stratum lacunosum-moleculare (LM) cell, and the interneuron-selective (IS) cell (reviewed in Freund and Buzsaki, 1996; Amaral and Lavenex, 2007). Chandelier cell bodies are located within the hippocampus proper and have dendrites that extend across all strata. In the rat, the axons of hippocampal chandelier cells synapse on the initial segments of approximately 1,200 pyramidal cell axons. Each pyramidal cell receives afferent input from 4-10 different chandelier cells. The basket cell bodies of the hippocampus proper are located in the pyramidal cell layer and the dendrites extend across all strata and receive excitatory synaptic input from pyramidal cells. Each pyramidal cell that synapses on a basket cell contributes only one synapse and in turn, a basket cell has between 2 and 10 synapses on the soma and proximal dendrites of pyramidal cells and synapses on as many as 1,000 pyramidal cells. Bistratified cells have cell bodies located in the pyramidal cell layer with dendrites located in all strata except lacunosum-moleculare. Axons of bistratified interneurons

form up to 16,000 synapses on pyramidal dendritic spines and shafts located within stratum oriens and radiatum (reviewed in Freund and Buzsaki, 1996).

Horizontal and radial trilaminar cells have large cell bodies located in either stratum lacunosum-moleculare or oriens. Horizontal trilaminar cell dendrites run horizontally in stratum oriens with spines located predominantly on distal dendrites. In contrast to bistratified cells, radial trilaminar cell dendrites are located in stratum lacunosum moleculare. Axons of trilaminar cells synapse on cell bodies of pyramidal neurons in the pyramidal cell layer. Both horizontal and radial trilaminar cells have over 15,000 axon terminals (reviewed in Freund and Buzsaki, 1996).

The OLM interneuron cell bodies and dendrites are located within stratum oriens in CA1 and in stratum oriens, lucidum, and radiatum of CA3 and receive afferent input from recurrent collaterals of pyramidal cells. The axons of OLM interneurons synapse on pyramidal cell dendrites located in stratum lacunosum-moleculare (reviewed in Freund and Buzsaki, 1996). Similar to OLM cells, LM cell bodies are located within stratum lacunosum-moleculare near stratum radiatum. The dendritic tree of these cells is bitufted often with a horizontal orientation. The axon collaterals of LM interneurons typically synapse on dendrites of pyramidal cells in stratum lacunosum moleculare (reviewed in Freund and Buzsaki, 1996).

Interneuron-selective (IS) cells synapse preferentially on other interneurons rather than directly influencing pyramidal cell activity. IS-1 cell bodies are preferentially located in stratum radiatum, oriens, and pyramidale of CA1 as compared to CA3, and their dendrites are located predominantly in stratum radiatum. Their axons synapse on dendrites and somata of other IS-1 cells and basket cells. IS-2 cell somata are located in

stratum radiatum with dendrites that extend into stratum lacunosum-moleculare. The axons of IS-2 cells descend to stratum pyramidale and synapse onto interneurons that are responsible for inhibition of pyramidal cells in CA1 that receive Schaffer collateral input (reviewed in Freund and Buzsaki, 1996).

Afferent Input to the Rat Hippocampal Formation

The hippocampal formation receives afferent input from the entorhinal cortex via perforant path axons (Ramon y Cajal, 1893). The perforant path axons that synapse on dentate granule neurons arise from cells located in layer II of the entorhinal cortex. Axons from cells located in the lateral entorhinal cortex synapse on dendrites of dentate granule neurons located in the outer molecular layer (lateral perforant path). In contrast, axons from cells located in the medial entorhinal cortex synapse on granule cell dendrites in the middle molecular layer (medial perforant path). The inner molecular layer of the dentate gyrus receives afferent input from intrinsic commissural fibers (Amaral and Lavenex, 2007; Figures 2.1 and Figure 2.2A).

Additionally, perforant path axons from cells in layer II and III of the entorhinal cortex synapse on dendrites of pyramidal cells in stratum lacunosum-moleculare of CA3, CA2, and CA1 (Ramon y Cajal, 1893). Lateral perforant path axons from layer II of the lateral entorhinal cortex synapse on the most peripheral pyramidal cell dendrites in stratum lacunosum moleculare, whereas medial perforant path axons from layer II of the medial entorhinal cortex synapse on the deeper half of stratum lacunosum-moleculare in CA3 and CA2 (Amaral and Lavenex, 2007; Figures 2.1 and Figure 2.2B). In contrast, perforant path axons originating from cells in layer III of the entorhinal cortex synapse on

pyramidal cells in CA1. Those originating from layer III of the lateral entorhinal cortex synapse on pyramidal cell dendrites in stratum lacunosum-moleculare in the distal portion of CA1 closest to the subiculum, whereas those originating from layer III of the medial entorhinal cortex synapse on pyramidal cell dendrites in stratum lacunosum-moleculare in the proximal portion of CA1 closest to CA2 (Amaral and Lavenex, 2007; Figures 2.1 and Figure 2.2C).

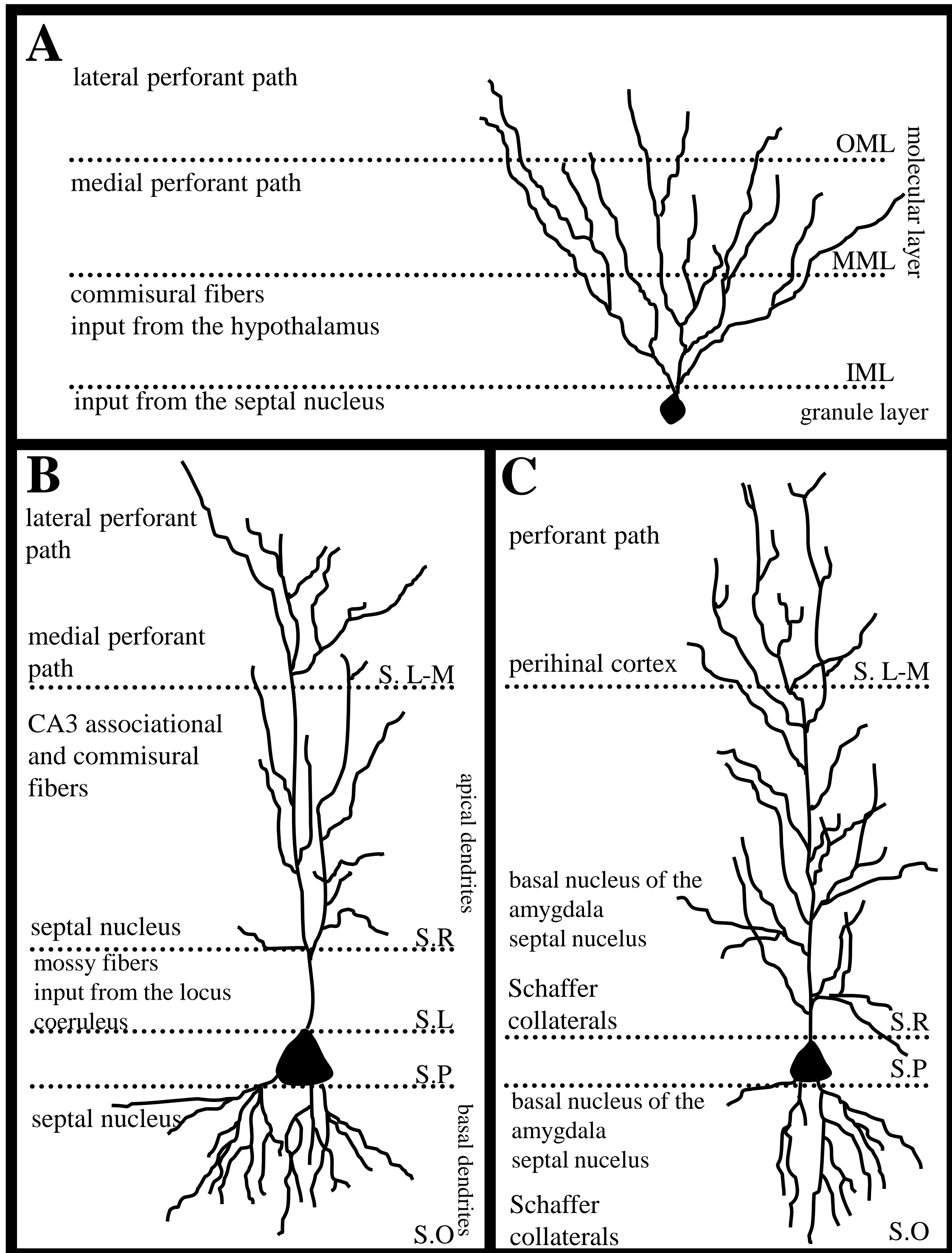

Figure 2.2: *Afferent input to principal cells of the hippocampal formation.* (**A**) granule neuron of the dentate gyrus. (**B**) pyramidal cells of CA3 and (**C**) CA1. List of abbreviations: OML: outer molecular layer, MML: middle molecular layer, IML: inner molecular layer, S.O: stratum oriens, S.P: stratum pyramidale, S.L.: stratum lucidum, S.R: stratum radiatum, S. L-M: stratum lacunosum-moleculare. Images of neurons are diagrammatic and are not neuronal reconstructions.

The dentate gyrus also receives afferent input from the presubiculum and parasubiculum (Kohler, 1985). The axonal fibers from cells originating in the subiculum synapse on dendrites of granule neurons at the border between the outer and middle molecular layer. These axonal projections are less extensive that that of the perforant path and fewer studies have focused on the function of these afferent fibers. None of the axonal projections from the presubiculum and parasubiculum synapse on cells located in the hippocampus proper (reviewed in Amaral and Lavenex, 2007).

In addition to the perforant path input (Figure 2.2), the hippocampal formation receives afferent input from other more distal regions of the brain. The hypothalamus and the septal nucleus provide afferent input to both the dentate gyrus and the hippocampus proper. However, some of the afferent inputs to the hippocampal formation synapse preferentially on cells within either the dentate gyrus or the hippocampus proper, and, importantly, do not synapse on cells in the other subregions of the hippocampal formation (Figure 2.2). These afferent inputs to the hippocampal formation will be described below.

Cells in the supramammilary area of the hypothalamus project to proximal dendrites of dentate granule neurons and are likely glutamatergic (Kiss et al., 2000). They also synapse on pyramidal cells in CA2, but not on neurons in CA3 or CA1 (reviewed in Amaral and Lavenex, 2007). In addition to cells in the supramammilary area, cells located in the lateral hypothalamus also synapse on dentate granule neurons.

The septal nucleus provides cholinergic and GABAergic afferent input to the dentate gyrus (Lubke et al., 1997; reviewed in Amaral and Lavenex, 2007). The majority of the cholinergic synaptic fibers originating from the septal nucleus that project to the dentate

gyrus synapse on mossy cells of the hilus; in contrast, the GABAergic input synapses on basket cells. In addition, CA3 pyramidal cells receive afferent input from the septal nucleus. Septal axons synapse on pyramidal cell dendrites located in stratum oriens and to a lesser extend in stratum radiatum. Pyramidal cells in CA1 also receive afferent input from the septal nucleus; however, a greater proportion of the septal axons to the hippocampus proper synapse on CA3 rather than CA1 pyramidal cells. Pyramidal cells located in CA3 and CA1 send axonal projections back to the septal nucleus. Interestingly, CA3 axon projections back to the septal nucleus are bilateral, whereas those from CA1 are only ipsilateral (reviewed in Amaral and Lavenex, 2007).

The brainstem also provides afferent input to the hippocampal formation. Neurons in the hilar region of the dentate gyrus receive noradrenergic afferent input from the locus coeruleus. Additionally, these noradrenergic axons synapse on dendrites in stratum lucidum of CA3. Serotonergic inputs from the dorsal raphe nuclei synapse on GABAergic neurons within the hilus, typically basket cells (reviewed in Amaral and Lavenex, 2007).

Neurons located within the perihinal cortex have axon projections that selectively synapse on the most distal CA1 pyramidal neurons (cells closest to the subiculum) and specifically on dendrites located in stratum lacunosum-moleculare. Additionally, distal CA1 pyramidal cells send axonal projections back to the perihinal cortex (reviewed in Amaral and Lavenex, 2007). The amygdala also provides afferent input to CA1. Axons from the basal nucleus of the amygdala synapse on dendrites of CA1 pyramidal cells located in stratum oriens and stratum radiatum, and CA1 pyramidal cells also send axonal projections back to the basal nucleus. None of the other subregions of the hippocampal

formation receives direct afferent input from the perihinal cortex or the amygdala (reviewed in Amaral and Lavenex, 2007).

Intrinsic Connections of the Rat Hippocampal Formation

Mossy fiber axons from dentate granule neurons synapse on i) cells within the hilus, ii) interneurons within the granule cell layer, and iii) pyramidal cells in CA3 of the hippocampus. The mossy fiber axons of granule cells enter the hilus and some local collaterals synapse on cells in the hilus (Claiborne et al., 1986; Ribak and Peterson, 1991). However, the main mossy fiber axons that project from granule neurons leave the hilar region and synapse on pyramidal cells of CA3 in the stratum lucidum layer as mossy fiber boutons (Claiborne et al., 1986).

In rats, mossy fiber axons are thin unmyelinated axons that are 0.1 to 0.7 μm in diameter with large synaptic terminals (Gaarskjaer, 1986). These terminals range from 3-5 μm in diameter and form synapses on large branched spines (thorny excrescences) located on the proximal apical dendrites of CA3 pyramidal neurons in stratum-lucidum. Interestingly, the location of a mossy fiber synapse in CA3 is correlated with the location of the dentate granule neuron that supplied the synaptic input (Claiborne et al., 1986). For example, the deepest mossy fibers are those in the infrapyramidal bundle, followed by the intrapyramidal bundle, and then the suprapyramidal bundle. In CA3c all of these bundles are prominent, whereas in Ca3b and CA3a the intrapyramidal and infrapyramidal bundle of mossy fibers are not typically observed. Mossy fiber axons from granule cells located in the infrapyramidal blade tend to comprise the infrapyramidal bundle; those from the crest of the dentate gyrus compose the intrapyramidal bundle; those from the

suprapyramidal blade make up the suprapyramidal bundle. Thus cells within the suprapyramidal blade of the dentate gyrus supply the majority of the synaptic input to CA3 pyramidal cells (Claiborne et al., 1986). Each mossy fiber axon has approximately 15 mossy fiber boutons that are on average 135 μm apart and that synapse on 15 separate CA3 pyramidal cells (Claiborne et al., 1986).

CA3 pyramidal cell axons form associational connections (CA3 to CA3) and Schaffer collateral connections (CA3 to CA1). CA3 axonal connections are widely distributed, yet still systematically organized (Cajal, 1901; Blackstad, 1956; Ishizuka et al., 1990, 1995; reviewed in Amaral and Lavenex 2007). The associational fibers project to synapse on ipsilateral and contralateral CA3 pyramidal neurons and interneurons located in stratum radiatum. Schaffer collateral axons that project from CA3 pyramidal cells synapse on dendrites of CA1 pyramidal cells in stratum radiatum, and to a lesser extend in stratum oriens (Ishizuka et al., 1990). In rats, a single CA3 pyramidal cell Schaffer collateral axon has 30,000 to 60,000 synaptic varicosities but with as few as 10 synapses between a single CA3 neuron and a single CA1 neuron (Sorra and Harris, 1993). Schaffer collateral axons provide 85% of glutamatergic synaptic input to dendrites of stratum radiatum and oriens of CA1 (Storm-Mathisen, 1977).

Hippocampal Commissure of the Rat

In the rat, there are both dorsal and ventral hippocampal commissures where axons from one hemisphere cross the midline to synapse on cells of the contralateral hippocampal formation, rather than synapsing on cells in the ipsilateral hippocampal formation (Blackstad, 1956; Frike and Cowan 1978; Laurberg, 1979; Wyss et al., 1980;

reviewed in Amaral and Lavenex, 2007). Those arising from the hilus compose the ventral hippocampal commissure, whereas those arising from the CA3 region compose the dorsal hippocampal commissure (Wyss et al., 1980).

In addition to providing afferent input to CA3 pyramidal cells via mossy fiber axons, dentate granule cells also project to synapse on granule cells in the contralateral hemisphere. Those axons synapse on the inner molecular layer of dentate granule neurons (Frike and Cowan, 1978). In addition to the Schaffer collateral and associational axon projections within the ipsilateral hippocampus, CA3 cells also project to the opposite hemisphere to synapse on CA3, CA2, and CA1 pyramidal neuron dendrites located in stratum radiatum and oriens of the contralateral hemisphere (Blackstad, 1956; Frike and Cowan 1978; Voneida et al., 1981; Buchhalter et al., 1990; reviewed in Frotscher and Seress, 2007). CA3 cells also receive afferent contralateral input from hilar neurons. CA1 neurons also project to synapse on cells in the contralateral CA1 region and on cells in the contralateral subiculum and entorhinal cortex (Blackstad, 1956; Frike and Cowan 1978; Voneida et al., 1981; Buchhalter et al., 1990; reviewed in Frotscher and Seress, 2007). It is important to note that hippocampal commissural connections are less abundant in primates as compared to rats (reviewed in Frotscher and Seress, 2007).

Efferent Projections of the Rat Hippocampal Formation

The majority of efferent axon projections from the hippocampal formation originate from pyramidal cells in CA1 and either project to synapse on cells in layer III of the entorhinal cortex or to cells in the subiculum. Proximally located CA1 pyramidal cell axons project to cells in the distal portion of the subiculum, whereas the axons of distally

located CA1 pyramidal cell axons synapse on more proximal cells in the subiculum (reviewed in Amaral and Lavenex, 2007). To a lesser extent, CA1 pyramidal neurons also project to the tenia tecta, medial frontal cortex, anterior olfactory nucleus, olfactory bulb, septal nucleus, basal nucleus of the amygdala, and the anterior and dorsomedial hypothalamus (van Groen and Wyss, 1990; Jay and Witter 1991; Amaral and Witter 1995; Kishi et al. 2006).

Development of the Rat Hippocampal Formation

The development of the hippocampal formation proceeds along specific gradients. Neurogenesis first occurs in the hippocampus proper followed by the dentate gyrus (Figure 2.3), with glia and interneurons being born before principal cells (Schlessinger et al., 1975, 1978; Bayer et al., 1980a, 1980b; reviewed in Frotscher and Seress, 2007). Within the hippocampus proper, CA3 neurons are born before those in region CA1; however, CA1 pyramidal cells migrate to their final destination before those of CA2 and CA3. Within the dentate gyrus, neurons in the suprapyramidal blade are born before those of the infrapyramidal blade.

Following neurogenesis, new neurons must migrate from the germinal layer to the appropriate subregion of the hippocampal formation and mature (reviewed in Frotscher and Seress, 2007). This growth and maturation of newly generated neurons requires dendritic and axonal outgrowth to ultimately form synaptic connections between subregions of the hippocampal formation. Axonal pathfinding requires the recognition of specific target layers and the formation of synapses. During hippocampal development,

afferent axons project to distinct layers and establish specific synapses with target cells (Figure 2.4).

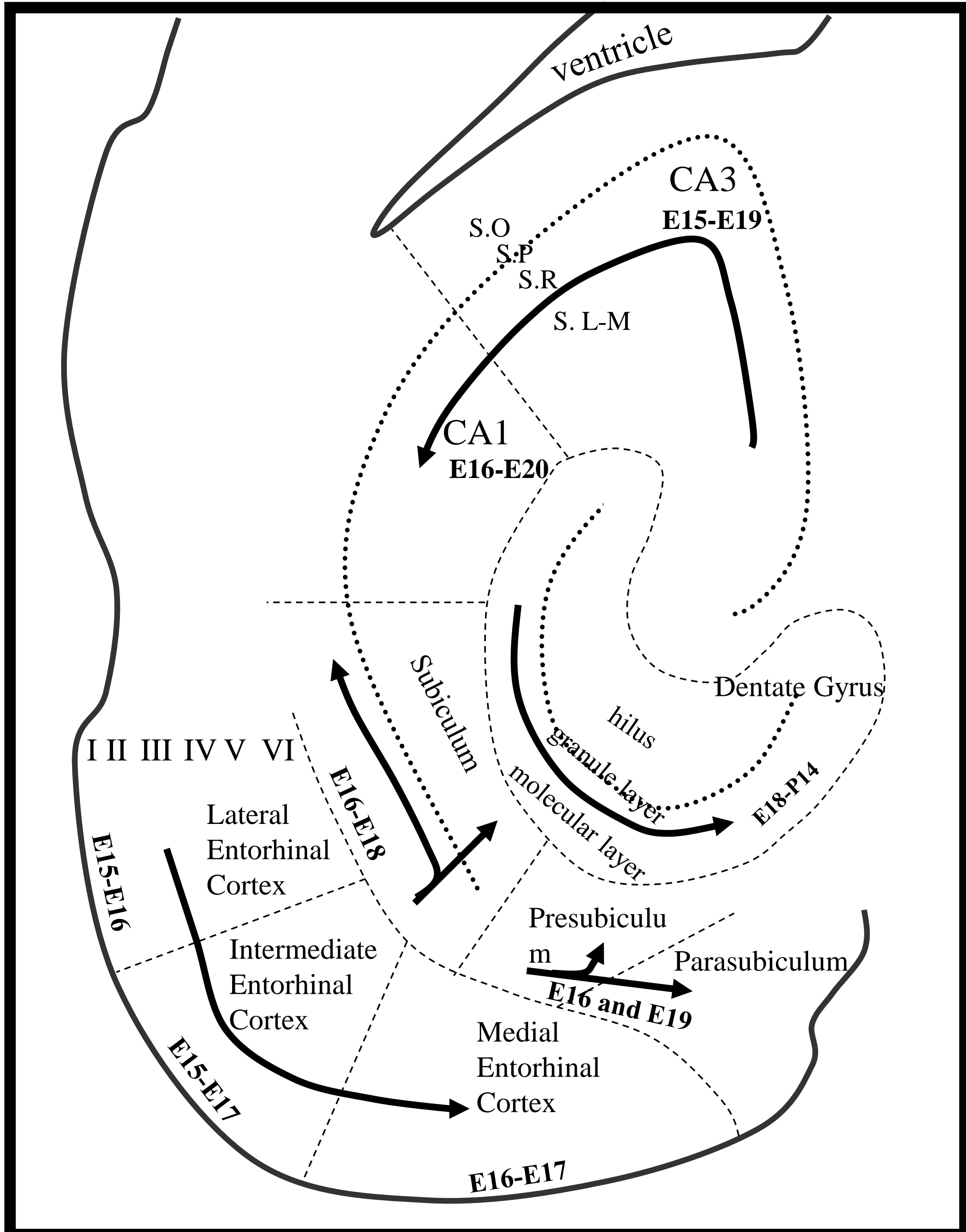

Figure 2.3: *Neurogenesis in the hippocampal region.* The gradient of neurogenesis for each subregion is denoted by an arrow. with the embryonic (E) or postnatal (P) day of neuronal birth indicated. Dates of neuronal birth and patterns of migration are from Schlessinger (1978) and Bayer (1980a). List of abbreviations: S.O: stratum oriens, S.P: stratum pyramidale, S.R: stratum radiatum, S. L-M: stratum lacunosum-moleculare.

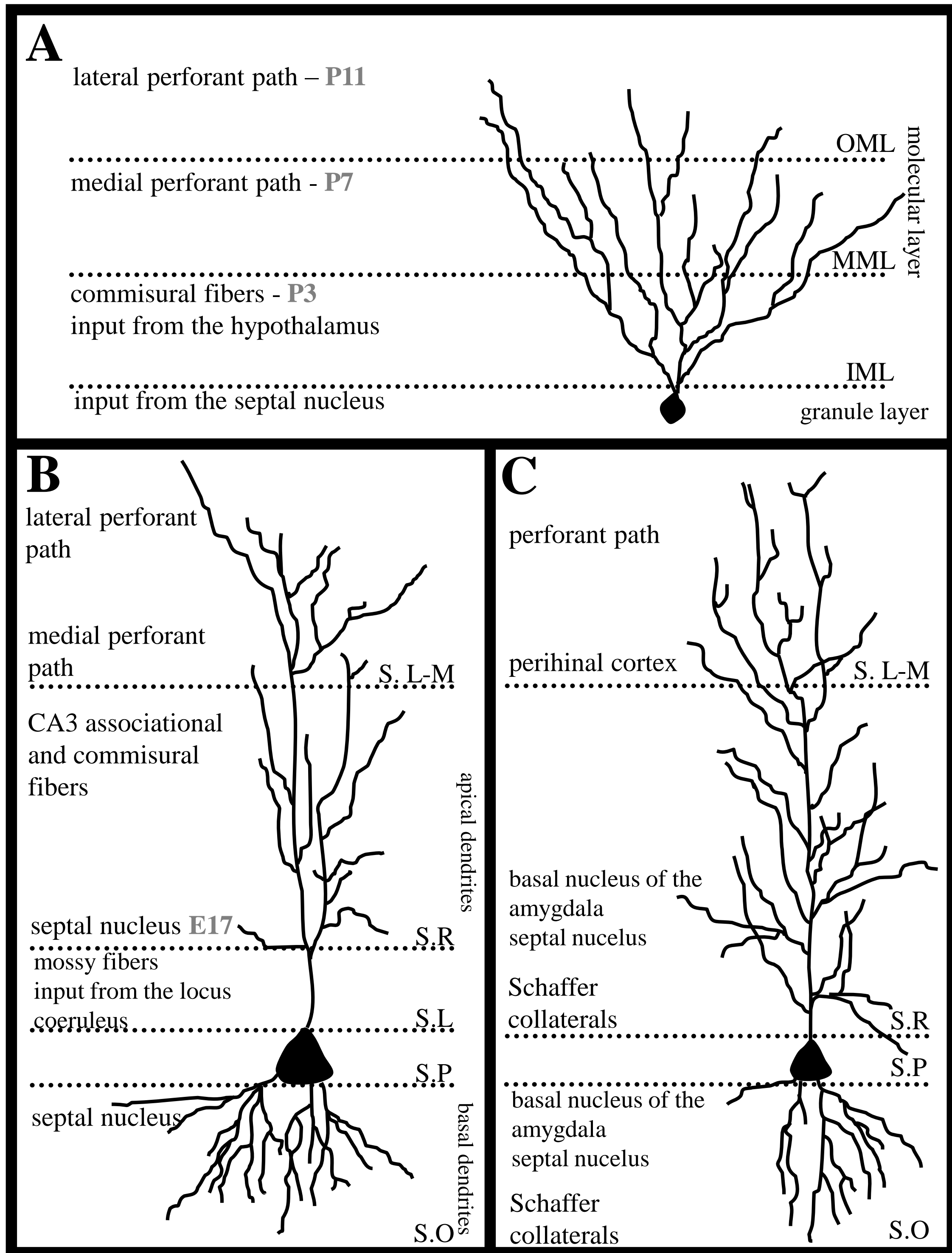

Figure 2.4: *Development of afferent inputs to principal cells of the hippocampal formation.* (**A**) granule neuron of the dentate gyrus. (**B**) pyramidal cells of CA3 and (**C**) CA1. List of abbreviations: OML: outer molecular layer, MML: middle molecular layer, IML: inner molecular layer, S.O: stratum oriens, S.P: stratum pyramidale, S.L.: stratum lucidum, S.R: stratum radiatum, S. L-M: stratum lacunosum-moleculare. (**Data for Timeline of afferent input to the hippocampal formation from** Tamamaki, 1999; Linke and Frotscher, 1993)

Development of the Rat Hippocampus Proper

New neurons in the hippocampus proper are born beginning in the CA3 region of the hippocampus on E14 and neurogenesis continues until E20 during late embryogenesis. Glia and interneurons are born prior to the pyramidal cells of the hippocampus proper in all subregions. Cell proliferation in the hippocampus proper includes glia, interneurons, and pyramidal cells (Bayer, 1980a, 1980b). The maturation of these cells and the synaptic connections formed between cells continues until adulthood.

Development of Glia in the Rat Hippocampus Proper

Gasser and Hatten (1990) have argued that glia have a role in early neuronal migration during rat hippocampal development in which neurons migrate along glial processes. Between E16 and E18, they found that fewer than 5% of the cells were labeled with glial filament protein (GFP) and those cells only included the cell body with few, short processes. By E20 the total number of glial cells increased to 15% of the total number of cells. The oldest glial cells at E20 had processes that were 120 µm in length and those cells began to clearly resemble radial glia. In addition to the more mature radial glial cells, smaller cells with very few short processes (30-50 µm) and other cells with 3 or more short processes (30-50 µm) were also observed at E29. Interestingly, Gasser and Hatton (1990) used microcultures of embryonic and postnatal hippocampal cells from Sprague-Dawley rats to show that when glia were cultured alone they remained relatively undifferentiated and continued to proliferate. However, when neurons and glia were cultured together, glia stopped proliferating and instead began to increase the extension

of their processes. These findings indicate that glia may have an important role in the development of the hippocampal formation.

Development of Interneurons in the Rat Hippocampus Proper

Interneurons are born prior to the principal cells in the hippocampus proper (Bayer, 1980). Amaral and Kurz (1985) utilized ^{3}H-thimidine labeling combined with immunohistochemistry for glutamic acid decarboxylase (GAD) and found that GABAergic interneurons were born at E14. However, interneurons do not migrate to the hippocampus proper until E16 and are not mature until after birth of the rat at E23/P0 (reviewed in Danglot et al 2006). In addition to the most commonly discussed and studied GABAergic interneurons, interneurons of the hippocampal formation have been shown to synthesize acetylcholine. In addition to synthesizing neurotransmitters, interneurons of the hippocampal formation can also synthesize neuropeptides (somatostatin, cholesystokinin, neuropeptide Y, vasoactive intestinal polypeptide, enkephalin, substance P, and neurokinin), neurotrophic factors (nerve growth factor, neurotrophin-3, and brain derived neurotrophic factor), calcium binding proteins (calbindin, parvalbumin, calretinin), and gases (nitric oxide). The neuropeptides produced by these interneurons do not easily classify them into types, as several types have been shown to produce the same neuropeptide (reviewed in Freund and Buzsaki, 1996; Danglot et al., 2006). These diverse cell types indicate that the birth, migration, and maturation of hippocampal interneurons must be tightly controlled.

Lang and Frotscher (1990) showed using Golgi staining of all interneuron types within the hippocampus that at P0 interneurons had large cell bodies with few, short

dendrites. By P5 the dendritic and growth cone length had increased; however, the interneurons of CA3 were more mature with dendrites that transversed several layers whereas those in CA1 had much shorter dendrites that were restricted to the layer of the cell soma. Thus, interneurons in CA3 were born first and differentiated earlier than those of CA1. Lang and Frotscher further showed that by P20 hippocampal interneurons showed all of the structural characteristics of adult interneurons.

Development of Rat Hippocampal Principal Cells

Pyramidal cells of the hippocampus proper and granule cells of the dentate gyrus originate in the ventricular epithelial (germinal) layers. In rats, pyramidal neurons are born between E15 and E21 with neurons of CA2 and CA3 being born (neurogenesis peaks at E17) before those of CA1 (peaks at E18 and 19; Bayer 1980). Although pyramidal cell neurons within CA2 and CA3 are born first, they must migrate 3-4 days longer than pyramidal cells destined to reach their final destination in CA1 (Altman and Bayer, 1990b). As a result, the CA1 neurons are born later but establish a distinct cell layer in stratum pyramidale of CA1 before CA3 pyramidal cells.

In the rat, the apical and basal dendrites of pyramidal neurons have begun to develop at birth (Loy, 1980). The length of the apical cell layer, which includes stratum lucidum, stratum radiatum, and stratum lacunosum-moleculare averages 441 μm at birth and reaches 88% of adult levels by P26 (872 μm) with the greatest acceleration of growth occurring between P0 and P10 (Loy, 1980). The width of the basal dendritic layer, stratum oriens, is approximately 210 μm (Loy, 1980). Although lamination of the hippocampal formation is established by birth, it continues to change and remodel until

adulthood (Loy, 1980); afferent innervation of the hippocampus proper will be discussed in greater detail in the following section.

In the rat, Gomez-Di Cesare and colleagues (1997) used biocitin to label CA3 pyramidal cells and found that CA3 pyramidal cells axons were short with few branches through the first postnatal week. However, by the end of the second postnatal week, axons of CA3 pyramidal cell axons were much longer. The total axon length was similar to that observed in adults, but with more extensive branching. Their findings suggested that, during early postnatal development, CA3 pyramidal recurrent axons continue to grow in length and to be remodeled until adulthood.

Commissural/associational fibers in CA3 are comprised of CA3 pyramidal axons that synapse on other CA3 pyramidal cells. In the rat, the commissural/associational synapses are not observed until P4 in CA3 in a 268 μm wide band in stratum radiatum that later expands to 514 μm at P26 (Loy et al., 1980). The commissural/associational pathways develop first in CA3b and mature outward from CA3b to CA3c and CA3a (Loy, 1980). Thus, the commissural/associational synapses are some of the latest to develop in CA3.

Development of Afferent Input to the Rat Hippocampus Proper

Afferent input to the hippocampus develops during embryonic development as the majority of afferent inputs to the hippocampus proper are already observed at birth (Loy, 1980). Bayer (1980) has suggested that the timing of afferent input to the rat hippocampus is related to the timing of cytogenesis in the region that provides that afferent input. For example, cells in the lateral entorhinal cortex are born before those of the medial entorhinal cortex (Bayer, 1980a) and when examining synapse formation in

the hippocampus proper, Loy (1980) found that lateral perforant path synapses developed prior to those of the medial perforant path. Furthermore, perforant path axon input to CA3 developed prior to the perforant path projections to CA1 (Loy, 1980). Findings such as these led Loy (1980) to argue that synapse formation in the hippocampal formation may not only depend upon the timing of the birth of neurons in regions that supply afferent input to the hippocampal formation, but also the time at which the hippocampal cells are competent.

Perforant path axons provide afferent input to the cornu amonis regions and synapse on dendrites in stratum lacunosum-moleculare of CA3 and CA1. In the rat, the lateral perforant path axons project to CA3 pyramidal neurons in the outer 211 μm of stratum lacunosum-moleculare at birth and this expands to 287 μm of stratum lacunosum-moleculare by P26 as the number of perforant path synapses increases during development and maturation as the CA3 pyramidal cell dendritic length increases (Loy, 1980). Perforant path axons synapse on the outer 78 μm of stratum lacunosum-moleculare of CA1 at P0, and this lamination expands to 140 μm at P10 (Loy, 1980). Thus, although perforant path synapses are present at birth, they continue to increase in number throughout the development of the hippocampal formation.

Dentate granule neurons also provide afferent input to hippocampal pyramidal cells via mossy fiber axon projections (Claiborne et al., 1986; Gaarskjaer et al., 1986). The oldest granule neurons begin to form mossy fiber axons prenatally and likely takes place once granule cells arrive in the granule layer (Gaarskjaer, 1986). Mossy fibers are observed exiting from granule cells in the suprapyramidal blade and reach the pyramidal cells of CA3 by P1. By P24, all dentate granule neurons have mossy fiber axons that

project to synapse on spines in stratum lucidum of CA3 pyramidal cells (Gaarskjaer, 1985, 1986). The mossy fiber axons of granule neurons elongate over the first two postnatal weeks, and after P14, the fibers regress to adult lengths by P28 (Amaral, 1979).

Development of the Rat Dentate Gyrus

Although the rat dentate gyrus begins to develop during late embryogenesis, the majority of cells proliferate, migrate, differentiate, and form synapses during postnatal development. Cell proliferation in the rat dentate gyrus includes dentate interneurons, mossy cells, and granule cells. New neurons in the dentate gyrus are born beginning at E14 with interneurons being born prior to principle cells (Schlessinger et al., 1978; Bayer, 1980a; Bayer, 1980b). These cells must then mature over the course of development, which typically continues to P60 when dendritic trees of dentate granule neurons are completely mature (reviewed in Rahimi and Claiborne, 2007). However, it is important to note that neurogenesis in the dentate gyrus continues to a lesser extent in the adult (Altman and Das 1975; Kaplan and Hinds, 1977).

Development of Rat Dentate Glial Cells

Radial glial cells are first observed at E15 in the rat dentate gyrus (Rickmann et al., 1987). From E15 until birth, they continue to develop and migrate to their final destination just below the granule cell layer. In a study of glial cell differentiation in the rat hippocampal formation, del Rio and colleagues (1991) examined GFAP expression in transverse slices of the hippocampal formation of rats from P0 to P5. They also used BrDU labeling to study neurogenesis and found that a large number of glial cells at all

ages studied had morphologies most closely resembling astrocytes with radial glial scaffolding present. Interestingly, del Rio and colleagues (1991) also found that glial cells in the hippocampus proper were much more developed during the first postnatal week as compared to those in the dentate gyrus.

Development of Rat Dentate Interneurons

Dentate gyrus interneurons are born prior to the principal cells in the dentate gyrus (Bayer, 1980). In rats, they are born prior to E19. Lubbers and colleagues (1985) utilized ^{3}H-thymidine labeling combined with immunostaining for GAD to determine the timing of the birth of interneurons that are observed in the adult (P40) rat. Of the total number of GABAergic interneurons labeled at P40, approximately 13% of those were born at E14. That percentage decreased to near 2% on E17 and 1% on E18 and none were observed at E19. Their findings indicate that interneurons of the dentate gyrus are first born during embryonic development and that the level of interneuron neurogenesis rapidly decreases before birth. However, the total percentage of adult interneurons born between E14 and E19 accounted for only 16% of the total; this led Lubber and colleagues to suggest that the majority of interneurons observed in the adult dentate may actually be born after P0.

Using Golgi staining, Seress and Ribak (1990) found that basket cells at P2 in the rat dentate gyrus, displayed immature dendritic features and small axon growth within the granule cell layer. By P5, basket cells in the dentate had formed synapses with granule cells. Seay-Lowe and Claiborne (1992) later more closely examined the dendritic trees and axon collaterals of interneurons in the suprapyramidal blade of the developing rat dentate gyrus at the end of the first postnatal week. The majority of dentate interneurons

observed at this age were immature GABAergic basket interneurons. Those cells had spines present on the cell body and dendrites and growth cones were still visible on some dendrites and axons. Many apical dendrites reached the top of the molecular layer and the basal dendrites extended as far as the CA3 pyramidal cell layer, which is not observed in the adult. Thus, the dendritic and axon arbors were extensive and still may represent immature features where regression would occur with continued maturation. Seress and Ribak found that basket cells in the dentate gyrus did not display adult-like mature features until P16.

Development of Mossy Cells in the Rat Hilus

In the rat, hilar neurons are born in the lateral and medial ganglionic eminence between E15 and E21 (Bayer, 1980; Seress and Ribak, 1983; Pleasure et al., 2000) and neurons born in the ganglionic eminence may account for granule cell neurogenesis that persists in the adult dentate gyrus (Altman and Das, 1965). Although hilar neurons are born during late embryonic development in the rat, mossy cells of the dentate gyrus continue to develop until the end of the third postnatal week (Ribak et al., 1985).

Mossy cells of the dentate gyrus are immature at birth, and their development lags behind principal cells of the hippocampus (Ribak et al., 1985). On P1, mossy cells have an immature appearance with dendritic growth cones that have filopodia as long as 10 μm without any typical spines or thorny excrescences that are present on mature mossy cells. The first adult-like spines are observed on proximal mossy cell dendrites at P7, but mature spines are not observed on distal dendrites until P14. Thorny excrescence spines

on mossy cells are not commonly observed until P14. Mossy cell proximal dendrites begin to resemble adult mossy cells on P21 (Ribak et al., 1985).

Adult mossy cell axons project to the contralateral dentate gyrus. Cowan and colleagues (1980) found that commissural axon projections to the contralateral dentate gyrus are present at the day of birth (P0) and at P1 small axon terminals of mossy cells are present. while, Ribak and colleagues (1985) found that mossy cell axons do not have a mature appearance until P14.

Development of Granule Cells of the Dentate Gyrus

The first dentate granule neurons are not born until E18, only 15% of dentate granule neurons are born prior to the birth of the rat (E22/E23; Bayer, 1980), and granule cell neurogenesis continues into adulthood (Altman and Das, 1965; 1966; 1967; Kaplan and Hinds 1977). Once dentate granule neurons are born, they migrate from the ventricular germinate layer through stratum oriens of the hippocampus proper to form the granule layer of the dentate gyrus. Neurogenesis of granule neurons in the dentate gyrus follows three gradients: i) septotemporal, ii) suprapyramidal to infrapyramidal blade, and, finally, iii) superficial to deep within stratum granulosum. Thus, the oldest granule neurons are those in the superficial layer of stratum granulosum in the suprapyramidal blade of the most septal part of the dentate gyrus (reviewed in Rahimi and Claiborne, 2007).

Neurogenesis of granule neurons in the dentate gyrus continues over a prolonged period in development, and even into adulthood (Altman and Das, 1976, Kaplan and Hinds, 1977). For this reason, at any point in early development a wide-range of granule neurons at varying levels of neuronal maturation are observed. The neurogenesis

gradients in the hippocampal formation may account for the differences in granule cell morphology observed in the adult rat (Claiborne et al., 1990). For example, neurons with cell bodies located within the superficial layers of stratum granulosum in the suprapyramidal blade have a greater number of primary dendrites, maximum branch order, transverse spread, elliptical spread, and percentage of dendritic length in the outer molecular layer as compared to granule cell neurons in deeper layers of stratum granulosum of the suprapyramidal blade. Thus, it appears that granule neurons born first have much more extensive dendritic trees than those born later.

At birth (E22/23, P0), the infrapyramidal blade is barely visible and grows more slowly than the suprapyramidal blade over the first postnatal weeks. At P0, the oldest granule neurons are already at least 5 days old at birth, but have only very rudimentary dendritic trees (Jones et al., 2003). By P3, the oldest dentate granule neurons exhibit at least one primary apical dendrite with short higher order branches. No filopodia or spines are present at this age. Basal neurons are also commonly observed on dentate granule neurons at P3, although these rudimentary features regress in adult granule cells. At P4, granule cell dendrites have filopodia (at least 10μm in length) and large varicosities with abrupt changes in dendritic diameter at dendritic branch points. Basal dendrites are still observed at this age. By P6 the number of filopodia has increased, and the abrupt diameter changes in dendrites and the presence of basal dendrites are reduced as compared to P4 in the oldest granule neurons. At P6 the average spine density on dendrites of dentate granule neurons in the middle molecular layer was 0.57spines/μm (Jones et al., 2003).

The first adult-like dentate granule neurons are observed at P7 (Desmond and Levy, 1985; Jones et al., 2003). At this time, the oldest granule neurons at P7 have dendrites that reach the top of the molecular layer, they no longer exhibit abrupt diameter changes and the number of filopodia has drastically decreased in comparison with the number of adult-like spines (0.81 spines/μm in the middle molecular layer; Jones et al., 2003) In addition, the basal dendrites are no longer present (Desmond and Levy, 1985; Jones et al., 2003). As the earliest granule neurons are born at E18 (Bayer, 1980), and the first adult-like neurons are not observed until P7 (Jones et al., 2003), then these findings would indicate maturation of dentate granule neurons takes at least 12 days from the birth of the neuron.

The presence of adult-like dentate granule neurons at P7 coincides with the time point in development when synaptic plasticities (LTP and LTD induction) are first observed in the dentate gyrus following stimulation of perforant path axons from the entorhinal cortex (Wilson, 1984; O'Boyle et al., 2004). Although adult-like dentate granule neurons are first observed at P7, the dendritic trees of those neurons continue to elongate and mature until the rat reaches young adulthood at P60 (1.66 spines/μm in the middle molecular layer; Desmond and Levy, 1985) that corresponds to the increase in the width of the molecular layer (Loy et al., 1977; Desmond and Levy, 1985; Jones et al., 2003; Rhin and Claiborne, 1990).

Rhin and Claiborne (1990) found that although the total dendritic length of granule neurons at P14 is equal to that of an adult rat, the molecular layer continues to widen until adulthood, also, branch order decreases after P14 whereas the length of lower order dendritic branches becomes longer total dendritic length (Rhin and Claiborne, 1990).

Rahimi and Claiborne (2007) proposed that changes in granule neuron structure between P14 and adulthood may result from changes in sensory input to the hippocampal formation via perforant path activity that occurs at the same time point in development.

Development of Afferent Input to the Dentate Gyrus

Afferent input to the rat dentate gyrus develops after birth in the rat. Using electron microscopy (EM), Cowan and colleagues (1980) found only a few synapses in the molecular layer of the suprapyramidal blade at P1 (0.4 synapses/100 μm^2). They also showed that the synaptic density increased by P4 (0.4 synapses/100 μm^2) and again by P10 (11 synapses/100 μm^2). In contrast, synapses in the infrapyramidal blade were not observed until P5 (0.8 synapses/100 μm^2) and increased by P10 (6.1 synapses/100 μm^2). The width of the molecular layer of the dentate gyrus also increased fourfold between P5 and P10 (Cowan et al., 1980). Thus, the afferent synaptic input to the dentate gyrus rapidly developed from P4 to P10.

Tamamaki (1999) found that afferent input to the dentate gyrus was first observed on proximal dendrites in the inner molecular layer and, lastly were observed on the most distal dendrites of the outer molecular layer of the dentate gyrus. Tamamaki used DiI to label neurons in the entorhinal cortex and hippocampal formation during postnatal development beginning at P3. Axons from the contralateral hilar region were discerned by P3 that synapsed on the inner molecular layer of the dentate gyrus, whereas the afferent axon fibers from the entorhinal cortex had yet to develop. Axons that originate in the medial entorhinal cortex that synapse on dentate granule neuron dendrites in the middle molecular layer were first observed at P7. Additionally, axons from the lateral

perforant path that originate in the lateral entorhinal cortex and synapse on dentate granule neuron dendrites in the middle molecular layer were first seen at P11 (Tamamaki, 1999).

Development of the Commissural Connections

Commissural projections are one of the last to develop in the hippocampal formation: the first commissural fibers synapses are observed at E18 in the rat hippocampus proper and at P2 in the rat dentate gyrus. Those commissural axons synapse on either proximal dendrites of pyramidal cells in stratum oriens and radiatum of the hippocampus proper or on dendrites of granule neurons in the inner molecular layer of the dentate gyrus (Avendano and Cowan 1979; Voneida et al 1981; Bayer and Altman, 1987; Buchhalter et al., 1990; reviewed in Frotscher and Seress, 2007). Although commissural synaptic connections are present at birth (Buchhalter et al., 1990), it has yet to be determined whether those synapses are mature.

Function of the Adult Rat Hippocampal Formation

Much of the knowledge of human hippocampal function in the human comes from studies of patients with memory impairments following lesions to all or part of the medial temporal lobe - the most famous of which were studies examining Henry Gustav Molaison (H.M; Scoville and Milner 1957). Following bilateral resection of H.M's medial temporal lobe (which includes the hippocampal region); Milner (Milner et al., 1968, 1972) noted that he had profound anterograde amnesia and slight retrograde amnesia. Although H.M. could recall his childhood, he had lost any memory starting 19

months prior to his surgery: he could no longer form new long-term memories. Thus, the medial temporal lobe, is not where long-term memories are stored, but plays a time-limited and critical role in the formation of new memories.

While the behavioral results of medial temporal lobe brain lesions indicate that memory impairments occur when those regions are damaged, they do not provide data that specifically isolate the hippocampal function. To more clearly discern the function of the hippocampal formation, and isolate each subregion of the hippocampal formation, animal studies have been utilized (reviewed in Rolls and Kesner, 2005). Selective lesions to different subregions of the hippocampal formation provide clear evidence of the role of each subregion in hippocampal function. Interestingly, these findings indicate that each subregion has a time-dependent role in learning and retrieval.

Dentate Gyrus

The dentate gyrus is thought to act as a completive learning network where place cells act to produce separate representations of a physical place (Rolls and Kesner, 2005). Specific bilateral lesions of the rat dentate gyrus resulted in a reduction in the ability to separate spatial patterns (Gilbert et al., 2001). Gilbert and colleagues tested rats in an object discrimination task. Rats were shown an object (A_1) and, following a short delay, rats were required to choose between two objects identical to the original object: one was in the exact same location as the original object (A_2) and the second object was in a different location (B) and the rat received a food reward when they chose the object in the same location as the original object (A_2). During the trials, the distance between A_2 and B ranged from 15 to 105 cm. Following bilateral lesions of the dentate gyrus, rats had

difficulties selecting the correct object (A_2) when the spatial separation between the two objects was small, but the rats were able to choose the correct object when the objects were further apart (105 cm). An additional study showed that rats with dentate gyrus lesions were less likely to explore changes in an environment when those changes were in close spatial proximity (Goodrich-Hunsaker et al., 2008). Rats naturally explore novel objects in an environment and, following habituation, those objects are ignored. If objects are moved following habituation, the objects are re-explored as novel. Following bilateral lesions to the dentate gyrus, rats were less likely to re-explore objects when they were in close spatial proximity, whereas, when they are moved further apart the rats did explore the objects as being novel. Thus, these previous findings indicate that the dentate gyrus is necessary for spatial pattern separation (Rolls and Kesner, 2006).

Further studies showed that the dentate gyrus is involved in the encoding, but not retrieval, of information (Lee and Kesner, 2004a, 2004b; Villareal et al., 2007). Lee and Kesner (2004b) tested rats in the Hebb-Williams maze where rats were required to navigate a maze based on the presence of extra-maze cues to obtain a food reward. Following colchicine-induced granule cell death (Goldschmidt and Steward 1990), rats made a greater number of mistakes during a single training day when they were tested in the Hebb-Williams maze (Lee and Kesner 2004b). Lee and Kesner (2004b) further tested rats using fear-conditioning and found that freezing behavior on the second day was reduced in rats that received colchicine-induced lesions of the dentate gyrus as compared to controls; thus, the rats with colchicine lesions were unable to learn to associate the tone (CS) with the footshock (UCS). These findings indicate that the dentate gyrus is necessary to learn these hippocampal-dependent tasks.

Hippocampus Proper

The CA1 and CA3 regions of the rat hippocampal formation are both involved in learning and recall; however, there are subtle functional differences between the two subregions. The CA3 subregion is involved in associational learning and rapid encoding of new information and is therefore important to novelty detection, pattern completion, and recall, especially from an incomplete set of cues or over a limited timespan. The CA1 subregion is more involved in sequence and order recall, particularly over time periods that last several days. Interestingly, while both CA3 and CA1 are involved in non-match to sample learning, if a delay between tasks is 10 seconds or less, then CA3 is necessary for completion of the task. In contrast, if the delay is at least 5 minutes, then CA1 is necessary to complete the task (reviewed in Rolls and Kesner, 2006).

The involvement of the CA3 region of the hippocampus proper in association learning and the recognition of patterns during recall is thought to be due to two factors. First, N-Methyl-D-Aspartate Receptor (NMDAR) dependent long-term potentiation (LTP) that allows for paired-associative learning. Second, and unique to CA3 of the hippocampal formation, the presence of recurrent associational and collateral fibers allows for re-activation of CA3 in associative learning and recall. Recurrent collaterals are also thought to be involved in pattern recognition during recall when a cue reactivates a subset of neurons thereby activating all of the neurons involved in the original encoding of the entire representation (reviewed in Rolls and Kesner, 2006).

CA3-dependent associate learning is thought to require NMDAR-mediated synaptic activity (Rajji et al., 2006; reviewed in Rolls and Kesner, 2006). Day and colleagues (2003) found that administration of APV, an NMDAR antagonist, to only CA3 of the

hippocampus blocked encoding, but not the recall of previously learned information. Furthermore, Rajji and colleagues (2006) showed that a focal deletion of the NR1 gene, which is necessary for NMDAR function, in CA3 of the rat hippocampus was sufficient to disrupt new learning, but does not affect the ability to complete a familiar paired-associate learning task that has already been learned. Day and colleagues (2003) also noted that treatment with CNQX, an AMPA receptor blocker, in CA3 blocked both the encoding and retrieval of information. Thus, unlike the NMDAR, AMPA receptor function in CA3 is necessary for both encoding and retrieval. Taken together, these data indicate that NMDARs in CA3 of the rat hippocampus appear to be specifically involved in learning.

The CA3 region is necessary for partial pattern completion, where an individual is able to complete a hippocampal dependent task when only a subset of cues is present (Gold and Kesner, 2005; reviewed in Rolls and Kesner, 2006). Gold and Kesner (2005) trained rats in a cheeseboard maze to obtain a food reward in a specific spatial location on the board based on the presence of extra-maze cues. Importantly, when less than half of the extramaze cues were available in later sessions, normal rats were still able to locate the food reward. However, following bilateral CA3 lesions, the rats were only successful at completing the task when more than half of the cues were present. Thus, when a sufficient number of cues were present in the environment it was recognized as familiar and the lesioned rats could still obtain the reward. In contrast, rats with bilateral CA3 lesions were not able to locate the reward when less than half of the extramaze cues were present. Therefore, the cues that remained no longer provided the context needed to determine the location of the food reward. This could indicate that the environment was

no longer recognized as familiar by rats with CA3 lesions, and may have been interpreted as a novel environment. Taken together, these findings suggest that CA3 is necessary for partial pattern completion and may be necessary for novelty detection (Gold and Kesner, 2005).

Villarreal and colleagues (2007) more closely examined the role of CA3 in novelty detection. During the initial exploration of a novel environment, CA3 commissural inputs in rats were attenuated on CA3 theta peaks, whereas perforant path inputs to CA3 were attenuated on CA3 theta troughs. These findings indicate that, in a novel environment, perforant path input to CA3 was favored on theta peaks and commissural input to CA3 was favored on theta troughs. Importantly, the theta-specific attenuation of afferent input to CA3 was absent when animals were re-exposed to the same environment, thereby indicating that habituation occurred and that changes in theta rhythm in CA3 are involved in novelty detection and, therefore, learning. Interestingly, administration of CPP, an NMDAR antagonist, did not block the attenuation of afferent input to CA3 on theta peaks and troughs when the rat was in a novel environment. In contrast, CPP did block the habituation that normally occurs when the rat was later presented with the same environment. Thus, habituation to an environment appears to be NMDAR-dependent, suggesting that at least some forms of learning are NMDAR dependent (Villarreal et al., 2007).

The CA1 region of the hippocampus is necessary for tasks that incorporate a time-delay and any task that requires the memorization of a sequence of events (Gilbert et al., 2001; Hunsacker et al., 2008; Kesner et al., 2010). For example, Gilbert and colleagues (2001) trained rats in the radial eight-arm maze by allowing them to explore the maze

randomly. Then only two arms were made available and the rat was required to choose the arm that occurred first in the sequence. Following CA1 lesions, the rats were less able to determine which of the arms came first in the sequence. Kesner and colleagues (2010) later showed that the memorization of a sequence of odor cues specifically required the ventral CA1, but not the dorsal CA1 of the rat hippocampus. Rats were trained to obtain a reward from a series of 5 scented cups presented in a temporal sequence; they were later required to choose the first in the sequence from two scented cups that were 2 of the 5 original cups. Following bilateral ventral CA1 lesions the rats were no longer able to distinguish the order in which the series of odor cues was originally presented (Kesner et al., 2010).

Conclusions

The timeline of the development of the hippocampal formation is potentially significant in considering the development of hippocampal lateralization. If lateralization of the hippocampal formation is observed during embryonic development, then those findings would indicate that hippocampal lateralization is established after the birth of glia (E16-E18; Rickmann et al., 1987; Gasser and Hatten 1990), interneurons (E14; Amaral and Kurz, 1985) and principal cells of the hippocampus proper (E15-E21; Bayer 1980), but before any of the hippocampal cell types are completely mature (Gasser and Hatten 1990) and before extensive connections between cells are observed (Gaarskjaer, 1985).

However, if lateralization of the hippocampal formation is not observed in embryonic development, then our previous findings of lateralized gene expression in the rat P6

(Moskal et al., 2006) would be the earliest indication that the development of the hippocampal formation is lateralized. Importantly this would indicate that lateralized gene expression in the hippocampal formation is observed after the birth of all cell types (Bayer 1980; Amaral and Kurz, 1985; Rickmann et al., 1987; Gasser and Hatten 1990), and only when the oldest cells are beginning to show mature features (Lubbers et al., 1988; del Rio et al., 1990; Jones et al., 2003; Desmond and Levy, 1985) and are first starting to form synaptic connections (Cowan, 1980; Lubbers and Frotscher, 1988; Tamamaki, 1999), but before the complete maturation of the hippocampal formation (Lubbers and Frostcher, 1988; Ribak et al., 1985 Seress and Ribak, 1990).

CHAPTER 3. HISTORICAL OVERVIEW OF BRAIN LATERALIZATION

Lateralization of the brain is often studied as the functional differences between the left and right hemispheres of the brain. However, more recently, lateralization of the brain has been studied at the physiological, anatomical, chemical, and molecular level in animal models in an effort to more clearly understand the mechanisms that underlie the establishment of lateralization and its functional significance in the adult brain. In the following chapter I will discuss some of the most significant studies in humans and rats related to the study of lateralization. First, I will discuss the earliest studies of lateralization in humans. Second, I will discuss the more recent studies of lateralization in humans that have focused on the changes in behavior following focused unilateral lesions to a particular brain region and the study of split-brain patients that no longer have connections between the left and right hemisphere. The focus of my dissertation is on lateralization of the rat brain; for this reason, I will also review the lateralization studies in rodents. It is important to note that a specific discussion of hippocampal lateralization in both the human and the rodent can be found in Chapter 4.

Lateralization in Humans

Early studies of lateralization in humans focused primarily on the functional lateralization of the brain associated with language (Broca 1861; Wernicke, 1881) and to a lesser extent on handedness (Annett, 1964; Oldfield, 1971; Annett 1972; Annett 1978; Hardyck and Petrinovich, 1977; Geshwind et al., 1978; Gut et al., 2007; Medland et al., 2009). More recent studies have focused on hemispheric asymmetry in “split-brain” patients (Sperry 1961; Bogen et al., 1965; Wilson et al., 1977; Gazzaniga et al., 1984),

which led to the utilization of brain imaging techniques to more clearly define hemispheric left-right differences in the normal human brain (Amunts and Zilles 2001; LeBihan, 2003). These findings are important as they suggest that lateralization is functionally significant in humans.

Lateralization of Language in Humans

The first indication that the brain might be lateralized in humans was the study of the preferential role of the left hemisphere in language in the 19th century. Broca (1861) and later Wernicke (1874) found that damage to specific regions within the left, but not the right, hemisphere resulted in specific behavioral changes related to speech and language comprehension. Broca's aphasia occurs when damage to the left inferior frontal gyrus (Brodmann's areas 44, 45, and 47) results in a loss of the ability to verbally communicate with no loss of the understanding of the speech of others: this is sometimes called an expressive aphasia (Broca, 1861; Brodmann 1905, 1909). In contrast, Wernicke's aphasia occurs when damage to the posterior superior temporal gyrus near the Sylvian fissure (Brodmann's areas 39 and 40) results in incoherent speech and an inability to understand the speech of others: this is sometimes called a receptive aphasia (Wernicke, 1874; 1910; Brodmann 1905, 1909; reviewed in Gannon, 2010). Importantly, the behavioral changes that occur as a result of damage to specific regions of the left hemisphere were the first to indicate that lateralized functional differences in language are also correlated with anatomical asymmetries.

In some instances language functions have been shown to be localized to the right hemisphere. These are not merely cases of crossed aphasia, where pronounced language

deficits are observed following lesions to the right hemisphere (Bakat et al, 1996), but rather more subtle changes in language that are observed even in individuals with language primarily located in the left hemisphere. Some individuals have difficulty distinguishing the intent of language and the tone of voice; for example, a stroke to the right parietal lobe may result in difficulty understanding sarcasm (Ross, 1981; Zatorre et al., 1992). These subtle changes in language following damage to the right hemisphere led Calvin and Ojemann (1994) to argue that the left hemisphere is necessary for grammar or the construction of language, whereas the right hemisphere is necessary to completely understand language.

Handedness

Handedness is one of the most obvious functional asymmetries in humans. Approximately 90% of the human population is right-handed (Annett, 1964; Annett 1972; Annett 1978). It is important to note that although taboos against left-handedness exist in many cultures (Falk 1980; Faurie and Raymond, 2004), a preferential use of the right hand has been detected as early as the first trimester in human fetal development (Hepper et al., 1991; Hepper et al., 1998; McCartney and Hepper 1999). Thus, any argument that asserts that the predominance of right-handedness is solely a result of cultural preferences may fail to account for hand preferences that are established prior to birth. However, the question as to whether the preferential use of one hand in manual tasks reflects a corresponding cerebral asymmetry remains largely unanswered.

As early as 1964, Annett suggested that handedness is controlled by a single gene that she termed the right-shift (RS) gene (Annett, 1964; Annett 1972; Annett, 1973; Annett

1978). Annett argued that dominant and heterozygous individuals would be right-handed (75% of the population), whereas recessive individuals (25% of the population) would be have no inherent predilection for either hand, but rather by chance would either be left or right handed (Annett, 1964). Based on Annett's model, roughly 12.5 % of the population would be left-handed, which is close to previously reported percentages of humans that have been shown to be left-handed (Oldfield, 1971; Hardyck and Petrinovich, 1977). However, despite the intervening 47 years, that gene has yet to be identified.

Although genetic screening approaches, including the use of microarray analysis combined with gene ontological analysis as described in Chapters 5 and 6 have led to the identification of genes that are differentially expressed during development (Sun et al., 2005; Moskal et al., 2006) and in the adult (Klur et al., 2009), identifying a single gene or genes, if they exist, that control handedness has proven difficult (reviewed in Corballis, 2010). In an excellent review of handedness, Corballis (2010) suggested that if Annett (1964; 1972; 1973; 1978) was correct in that chance plays an important role in the determination of left-handedness it would necessarily lead to difficulties in identifying a candidate gene, because a simple comparison of left-handed and right-handed individual's chromosomes would also include those individuals who are right-handed by chance.

Interestingly, it has previously been suggested that handedness and the lateralization of language are likely controlled by the same mechanism (reviewed in Corballis, 2010) where a left-handed individual would utilize the right hemisphere in speech and language comprehension – a reversal of the asymmetry that is typically observed. This argument is typically based on previous findings that suggest that language evolved from manual

gestures, rather than vocal calls (reviewed in Corballis, 2010). However, studies of individuals with aphasias produced as a result of a unilateral lesions to Broca's and Wernicke's area indicate that aphasias are still observed in 60% of left-handed individuals with damage to the left-hemisphere (Levy, 1971; Levy and Mandel, 1972; reviewed in Levy and Nagylaki, 1972; Naeser and Borod, 1986) and in some cases in right-handed individuals with damage to the right-hemisphere (Bakar et al., 1996). Importantly, these findings indicate that studies of handedness cannot be utilized as a way of indirectly studying directional preference in the asymmetry of the brain: there is simply not a *dominant* hemisphere in each individual. Rather, hemispheric specialization for a specific task or behavior in humans is more commonly observed (Sperry 1961; Bogen et al., 1965; Gazzaniga et al., 1984).

The Study of "Split-Brain" Patients

Roger Sperry and Michael Gazzaniga studied human patients that had undergone treatment for intractable epilepsy by having the corpus callosum (a large white matter tract that connects the left and right hemisphere of the brain) severed in an attempt to prevent the spread of seizures from one hemisphere to the other (Sperry 1961; Bogen et al., 1965; Wilson et al., 1977; Wilson et al., 1977; Gazzaniga et al., 1984). Severing the cerebral commissures limits the exchange of information between hemispheres. Interestingly, while in many cases these patients were seemingly no different from any other person, differences in behavior were observed upon closer examination. Their findings indicated that functional lateralization of other brain regions exist in addition to those known for speech production and language comprehension. They found that the

somatosensory, motor, and visual systems are functionally lateralized in split-brain patients (Gazzaniga et al., 1963; Gazzaniga, 2000).

In normal individuals, somatosensory information is processed in the dorsal-column-medial-lemniscus pathway or anterolateral pathway. In both of these pathways the second neuron in the pathway decussates (crosses over) to the opposite hemisphere. Thus, sensory information received by the left half of the body is processed by the right somatosensory cortex and sensory information received by the right half of the body is processed by the left somatosensory cortex (Martin, 2003). It is important to note that in most individuals the somatosensory information received by one hemisphere projects to the same region in the contralateral hemisphere via the corpus callosum; therefore, both hemispheres receive the same sensory information (Martin, 2003). However, in split-brain patients, one hemisphere no longer receives sensory input from the contralateral hemisphere. As a result, those patients only receive somatosensory information from the contralateral side of the body. In studies of the somatosensory system of split-brain patients, Gazzaniga and colleagues (1995) found that when sensory information must be integrated, split brain patients cannot accomplish the task. For example, patients cannot identify an object (e.g. a key) with their right hand when they have a similar object (another key) in their left hand (reviewed in Gazzaniga, 1995).

Split-brain patients can still use their hands and initiate motor actions in a seemingly coordinated fashion. However, upon closer examination, differences exist between split-brain patients and controls when complex motor tasks must be completed at the same time. In some instances split-brain patients are better able to complete tasks that require a dissociation of motor tasks to either hand. For example, split-brain patients can draw two

very separate images at the same time with both hands, whereas, normal control patients have greater difficulty with this task (Gazzaniga, 2000). Bogen and Gazzaniga (1965) have also shown that split-brain patients struggle to complete complex motor-tasks with their right-hand: split-brain patients can construct block designs with their left hand, but not their right. Furthermore, split-brain patients have difficulty controlling or coordinating their behavior when the two hemispheres disagree. For example, one patient would hold a book with his left hand while reading and his right hemisphere that controls left-arm and hand movement and is not involved in language or reading comprehension would command the left hand to throw the book away (Gazzaniga et al., 1962; reviewed in Gazzaniga 1995; 2000).

Visual sensory input is processed based on the location of that input in either the left or right visual field. Information about an image in the left visual field is processed by the right hemisphere and the right visual field by the left hemisphere (Martin, 2003). Gazzaniga and colleagues investigated the functional lateralization of the visual system in non-human primates (Nakamura and Gazzaniga, 1977), and in split-brain patients (Kroll et al., 2003; Gazzaniga et al., 1975). To isolate the left and right visual system of the macaque, Nakamura and Gazzaniga (1977) cut both the optic chiasm and the corpus callosum. If those split-brain macaques peered through an eye-hole that only allowed their left eye to receive visual input they could use that input to complete a task in order to receive a food reward, whereas the right eye and, therefore, the right hemisphere could not.

Similarly, in split-brain human patients when a word or image was projected to the right visual field (left hemisphere) the split-brain individual could identify the word or

image. When the word or image was projected to the left visual field they could not identify the word or object. However, when asked to pick out the word or object, or to draw the object with the left hand, they were successful without being consciously aware of having observed the object (Gazzaniga 1989; reviewed in Gazzaniga 1995; 2000; Wolford et al 2000). This observation led Gazzaniga to argue that the right hemisphere is the intuitive hemisphere, whereas the left hemisphere is the logical hemisphere (Gazzaniga 1987; Luck et al 1989; reviewed in Gazzaniga 2000).

Studies of Humans Following Unilateral Brain Damage

In addition to the study of split-brain patients mentioned above, studies of brain lateralization in humans have also focused on the effect of unilateral lesions in a single hemisphere following stroke (reviewed in Calvin and Ojemann, 1994). As I mentioned above, the most famous studies of unilateral lesions were those conducted by Broca (1861) and Wernicke (1881) regarding the localization of language to the left hemisphere. However, other studies have been directed toward the study of patients that have received unilateral lesions to other regions of the brain.

The study of lateralization following damage to the right hemisphere indicates that the most common result is unilateral neglect. In those cases of unilateral neglect an individual will ignore the left visual field, and, therefore, will only draw the right half of an image, only attend to movement in the left visual field, and in extreme cases will even neglect the left half of their body and no longer recognize it as their own (reviewed in Calvin and Ojemann, 1994). Additionally, the right hemisphere is thought to be necessary for facial expression recognition (Fried et al., 1982; Sergent et al., 1992). Findings such

as these have led some to argue that the right hemisphere is necessary for self-awareness and the understanding and expression of emotion (Calvin and Ojemann, 1994; Gazzaniga, 2000; Gazzaniga, 2005).

Lateralization in Rats

Despite the fact that the functional lateralization of the brain in humans has been studied since the late 19th century (Broca 1861; Wernicke, 1881), it has only been during the last several decades that hemispheric asymmetry has been studied in other species. Lateralization was once thought to be a uniquely human phenomenon, but more recent findings indicate that lateralization is observed across vertebrate species, thereby indicating an early origin of lateralization (reviewed in Rogers, 2004; Rogers, 2006; Vallortigara and Rogers 2005; Hughdahl and Westerhausen, 2010). Importantly, the study of lateralization in non-human species, such as the rat, allows for investigators to more closely examine the significance of lateralization to proper brain development and function.

In an effort to directly compare lateralization in humans and rodents, studies of paw preference in rodents have been utilized as a means of comparison to studies of human handedness (Pence, 2002; Sun and Walsh, 2006; Tang et al., 2008; Vyazovskiy and Tobler, 2007). Paw preference in rats is determined based on the paw most frequently used to reach food. Although paw preference is observed in individual rodents, it is not often observed at the population level (Waters and Denenberg, 1994; Bulman-Fleming et al., 1997; Guven et al., 2003; reviewed in Sun and Walsh, 2006) as handedness is in humans (Annett, 1970).As I noted above, the study of handedness has been criticized as a

means of studying lateralization in humans as it is not always directly correlated with differences in lateralization of the brain; this is also true in the study of paw preference in rodents (Denenberg 1983; Sun and Walsh, 2006). I will instead focus on a review of previous studies directed toward the study of the lateralized rat brain. As Chapter 4 focuses specifically on the study of lateralization of the hippocampal formation in both humans and rats, here I will focus on a more broad review of lateralization in the rat brain.

Lateralization of the Rat Brain

In an effort to understand the mechanisms that underlie the establishment of cerebral lateralization in the rat, others have focused on sex differences in the pattern of asymmetric cerebral development (Geshwind and Levitsky, 1968; Geshwind and Gallaburda 1985; Diamond 1991) leading to hypotheses that either glucocorticoids (Bakalkin, 1989; Sullivan and Gratton, 1998; Takahashi, 1996; de Kloet et al., 1999; Chen et al., 2001; Ordyan et al., 2001; Sullivan et al., 2004; Alfarez et al., 2008) or testosterone (Galaburda and Geschwind 1981; Geschwind and Galaburda 1985; Geshwind and Miller 2001; Geshwind and Galaburda 1985a, 1985b, 1985c; Grimshaw et al., 1995) are involved in asymmetric development. Sex differences in laterality can be observed as early as the first postnatal week (Ross et al., 1981; Diamond, 1991). Ross and colleagues (1981) used 2-deoxy-D-glucose to measure lateralized metabolic activity in the frontal cortex, hippocampus, diencephalon, and brainstem in male and female Sprague-Dawley rats daily from P0 to P7. Unfortunately, the authors pooled the data for each of the males and each of the females, so any individual fluctuations over the first

postnatal week were not determined. However, the authors did note that asymmetric 2-deoxy-D-glucose uptake was greater in the right hippocampus, right diencephalon, left cortex, and the left medulla and pons for the female, but not the male, rats. These findings indicate that metabolic lateralization is observed in female rats, but without a clear directional preference, and that male rats show no lateralization of glucose metabolism at all.

In a later study, Diamond (1991) examined sex differences in laterality of the cerebral cortex in Long-Evans rats. In males the cerebral cortex was thicker in the right hemisphere as compared to the left, whereas in females when significant differences between the two hemispheres were observed the greater volume was in the left hemisphere. Interestingly, the greater thickness of the right cerebral hemisphere was observed as early as P6 and was still observed after 2.5 years of age (P900) in male Long-Evans rats (Diamond 1988). Taken together, these findings could indicate strain differences in lateralization of the cerebral cortex (Ross et al., 1981; Diamond 1988; Diamond 1911).

Cooke and Woolley (2005) found sexually dimorphic asymmetry in the medial amygdala prior to puberty in the rat. Male rats had greater mEPSC frequency, but not mEPSC amplitude, in cells located in the left medial amygdala as compared to females. It is important to note that Cooke and Woolley (2005) did not observe any significant difference between the hemispheres for either males or females.

Although findings related to sex-differences in laterality are intriguing, the majority of studies directed toward lateralization in rats have focused exclusively on males. The potential role of glucocorticoids in the establishment of asymmetry has led many to

examine the role of stress on lateralization of the rat brain (Sullivan and Gratton 1998; Sullivan et al., 2004; Sullivan and Dufresne, 2006; Czeh and colleagues, 2008). Czeh and colleagues (2008) specifically examined the role of stress on lateralized anatomy of the medial prefrontal cortex (mPFC). Using Sholl analysis, Czeh and colleagues (2008) found that in control (non-stressed) rats lateralization of dendritic length was present, but very subtle: although total dendritic length was not lateralized in neurons located in the prelimbic cortex, dendrites in the middle and distal portions of the apical tree were longer in the right hemisphere as compared to the left. Furthermore, neurons in the infralimbic cortex had greater apical dendritic length more proximal to the soma. Following 21 days of immobilization stress, lateralization of dendritic branching was no longer observed: in the prelimbic cortex dendritic length decreased in the middle and distal regions of the apical tree in the right hemisphere resulting in a loss of the lateralization normally observed. Additionally, in the right infralimbic cortex total dendritic lengths decreased preferentially near the soma (Czeh et al., 2008).

Although Czeh and colleagues (2008) observed subtle differences in neuroanatomical lateralization in the mPFC, many other types of lateralization in the PFC show a clear directional preference. Slopsema and colleagues (1982) examined norepinephrine and dopamine in the male rat prefrontal cortex, and they found that dopamine levels were greater in the left medial prefrontal cortex as compared to the right. However, the authors did not observe lateralized levels of norepinephrine. Sullivan and colleagues (Sullivan and Gratton, 1998; Sullivan, 2004; Sullivan and Dufresne, 2006) observed greater dopamine levels in the left prefrontal cortex of the rat as well. Interestingly, they also showed that handling rats during the first three postnatal weeks resulted in greater

dopamine levels in the right hemisphere that was correlated with an increased ability to handle stress.

Pediconi and colleagues (1993) determined the M_1 and M_2 muscarinic acetylcholine receptor (mAChR) densities in dissociated cells from the left and right cerebral cortex of male rats and found that the M_1 mAChR density was 50% greater in the cells taken from the left cerebral cortex. Kristofikova and colleagues (2004; 2008; 2010) also observed lateralized high affinity choline uptake in the rat hippocampal formation (discussed in greater detail in Chapter 4). Additionally, aminopeptidase, an enzyme that acts to break down proteins by specifically breaking peptide bonds at the terminal end of the amine group, was greater in the left frontal cortex and hypothalamus of male rats (Alba et al., 1998).

While work on lateralization of the rat brain has been primarily directed toward the study of cerebral asymmetry, other regions of the brain, such as the basal ganglia are also asymmetric (Schneider et al., 1982; Capper-Loup and Kaelin-Lang 2008; Capper-Loup et al., 2009; Meitzen et al., 2011). For example, Schneider and colleagues (1982) utilized the binding of H^3spiroperidol, a radiolabeled D2 receptor antagonist, to label the number of D2 receptors in the striatum of adult male Sprague-Dawley rats. They found that the radiolabeled binding was 23% greater in the left striatum. Capper-Loup and Kaelin-Lang (2008) later examined the expression of dynorphin (DYN), glutamic acid decarboxylase (GAD), and enkephalin (ENK) mRNA in the medial and lateral striatum of adult female Sprague-Dawley rats. They found that DYN and GAD were differentially expressed, whereas ENK was not. This led Capper-Loup and Kaelin-Lang (2008) to conclude that the direct, but not the indirect, striatal pathway is likely lateralized. More specifically,

DYN and GAD were more highly expressed in the left medial striatum, but were not differentially expressed in the lateral striatum. The authors further concluded that afferent input from the limbic system may contribute to the lateralization of the medial striatum, whereas the afferent input to the lateral striatum from the sensorimotor cortices are not lateralized.

Capper-Loup and colleagues (2009) further examined the mRNA expression of the NR1, NR2A, and NR2B subunits of the NMDAR in the left and right medial and lateral striatum. They also examined the expression of the vesicular glutamate transporter 1 (vGluT1) in the limbic cingulate cortex, the medial agranaular cortex, and the primary motor cortex regions that all provide afferent input to the medial striatum, to determine whether the afferent input is also lateralized in female Sprague-Dawley rats. Capper-Loup and colleagues (2010) found that NR2A was more highly expressed in the left medial striatum, and was not lateralized in the lateral striatum. Furthermore, the NR2B and NR1 subunits were not differentially expressed in either the medial or lateral striatum. The vGluT1 receptor was more highly expressed in the left limbic cingulate cortex, but was not differentially expressed in the agranular or primary motor cortices. Combined with their previous findings (Capper-Loup and Kaelin-Lang 2008) these results suggest that afferent limbic input to the striatum and the direct pathway within the striatum that receives that afferent input are lateralized. Meitzen and colleagues (2011) later studied the lateralization of cell density in the dorsal striatum and the nucleus accumbens of Sprague-Dawley rats. Neuron density was greater in the left dorsal striatum and was not sexually dimorphic. However, it is important to note that neuron density was not lateralized in the nucleus accumbens of either male or female rats.

In addition to the above examples that indicate a left-hemispheric dominance in the response to stress (Sullivan, 2004; Sullivan and Gratton, 1998; Sullivan and Dufresne 2006, Czeh et al., 2008) and in regions of the brain that provide or receive afferent input from the limbic system (Capper-Loup and Kaelin-Lang 2008; Meitzen et al., 2011), other forms of lateralization have been observed to be right-hemisphere dominant (Robinson, 1975; Robinson and Coyle 1979; Perez et al., 1990). Robinson (1975) examined the asymmetric effect of middle cerebral artery occlusion (MCAO) on behavior. Following right MCAO, rats remained hyperactive for up to 3 weeks, norepinephrine was reduced in the locus coeruleus, and dopamine was reduced in the substantia nigra. Although left MCAO caused a similar amount of tissue damage it produced none of the behavioral or neurochemical changes (Robinson and Coyle 1979). Perez and colleagues (1990) later studied the physiological lateralization of the cerebral cortex in rats. Although they found no differences between cells of the left and right prefrontal cortex in response to a single stimulation, the authors did note asymmetries following paired stimulations. Following paired pulses the increase in amplitude was greater in the right hemisphere as compared to the same paired pulses in the left prefrontal cortex.

Summary

Previous findings indicate that lateralization of the brain is observed in multiple species, including humans and rats. Functional lateralizaton is most easily identified after unilateral brain injury. Although the case study approach is very informative in understanding some forms of lateralization in humans, it has the drawback of not being as

specific. The study of lateralization in animals, including rats, allows for the possibility of more closely examining the mechanisms that act to establish lateralization of the brain.

CHAPTER 4. LATERALIZATION OF THE HIPPOCAMPAL FORMATION

The hippocampal formation is known to be necessary for certain forms of learning and memory in humans (Scoville and Milner, 1957) and rodents (O'Keefe and Dostrovsky, 1971; Bliss and Lomo, 1973; Olton and Samuelson, 1976; Morris et al., 1982; reviewed in Squire 1992; Moser and Moser, 2008). The intense interest in understanding the mechanisms that underlie learning and memory has led to the hippocampal formation being the focus of innumerable studies over the last several decades. However, relatively few studies have been directed specifically toward the study of hippocampal lateralization (Diamond et al., 1982; Bernasconi-Guastalla et al., 1994; Tabibnia et al., 1999; Maguire et al., 2000; Poe et al., 2000; Tang, 2001; Zou et al., 2001; Verstynen et al., 2001; Kawakami et al., 2003; Kristofikova et al., 2004; Hanlon et al., 2005; Sommer et al., 2005; Lister et al., 2006; Moskal et al., 2006; Kawakami et al., 2008; Shinohara et al., 2008; 2009; Tang et al., 2008; Thompson et al., 2008; Klur et al., 2009; Samara et al., 2011) and only a small number have examined the early development of hippocampal lateralization (Moskal et al., 2006; Thompson et al., 2008).

In the human, functional, anatomical, and neurochemical asymmetries of the hippocampal formation have been observed (Glick and colleagues, 1982; DeLisi and colleagues 1989; Tranel, 1991; Abrams et al., 1997; Zaidel and colleagues 1997; Bohbot et al., 1998; Maguire et al., 1998; Kelley et al., 1998; Strange et al., 1999; Eldridge et al., 2000; Maguire et al., 2000; Spiers et al., 2001; Burgess, 2001, 2002; Hanlon et al., 2005; Simic et al., 2005; Sommer et al., 2005; Glickman-Johnston et al., 2008; Thompson and colleagues 2008; Binder et al., 2009; Fink and colleagues 2009; Mechanic-Hamilton et al., 2009; Barkas et al., 2010; Bonelli et al., 2010; Ulrich et al., 2010). Functional,

anatomical, neurochemical, and molecular asymmetries have also been documented in the rodent hippocampal formation (Diamond et al., 1982; Bernasconi-Guastalla et al., 1994; Poe et al., 2000; Tang, 2001; Verstynen et al., 2001; Kawakami et al., 2003; Kristofikova et al., 2004; Wu et al., 2005; Lister et al., 2006; Moskal et al., 2006; Tang et al., 2008; Shinohara et al., 2008; 2009; Klur et al., 2009; Samara et al., 2011). In this chapter I will first discuss the left-right differences observed in the human hippocampal formation, followed by a discussion of hippocampal lateralization in the rodent.

Lateralization of the Human Hippocampal Formation

The study of human hippocampal lateralization has been primarily concentrated on patients with parahippocampal lesions (Abrahams et al., 1997; Bohbot et al., 1998; Binder et al., 2009; Glickman-Johnton et al., 2008; Mechanic-Hamilton et al., 2009; Barkas et al., 2010; Bonelli et al., 2010) or the use of imaging techniques (Tranel, 1991; Eldridge et al., 2000; Hanlon et al., 2005; Sommer et al., 2005; Ulrich et al., 2010). However, postmortem (Zaidel and colleagues 1997; Simic et al., 2005) and MRI (Thompson et al., 2008) studies have also been conducted to examine lateralized anatomy of the hippocampal region. Furthermore, lateralization of specific neurochemicals has been studied in the human brain (Glick et al., 1982).

Functional Lateralization of the Human Hippocampal Formation

It has been shown that hippocampal function is lateralized following unilateral lesions of the parahippocampal cortex for the treatment of entractable epilepsy. Although unilateral parahippocampal lesions do not produce complete anterograde amnesia, as is

seen following bilateral lesions of the hippocampal formation (Scoville and Milner 1957; Milner, 1972; Corkin, 1984; Zola-Morgan et al., 1986), specific memory impairments are still observed (Abrahams et al., 1997; Bohbot et al., 1998; Binder et al., 2009; Glickman-Johnton et al., 2008; Mechanic-Hamilton et al., 2009; Barkas et al., 2010; Bonelli et al., 2010).

Patients treated for epilepsy using unilateral parahippocampal lesions show deficits in spatial memory tasks when the right, but not the left, hippocampal formation is lesioned (Bohbot et al., 1998). Bohbot and colleagues studied patients with focal ipsilateral lesions to either the left or right hippocampus and found that patients showed deficits in a version of the Morris water-maze task (MWMT) used on human subjects only when a delay was incorporated into the task. In the MWMT patients were put in a room where they utilized external spatial cues in order find a hidden sensor under the carpet that would indicate successful completion of the virtual maze. Following a 30 minute delay in the MWMT, patients with lesions to the right parahippocampal cortex showed memory impairments, whereas, those with lesions to the left parahippocampal cortex successfully completed the task. Thus, following a delay, the right parahippocampal formation was necessary for successful completion of the VMWMT.

Spiers and colleagues (2001) later utilized a virtual town similar to modern first-person computer games to assess performance in a maze where a subject is asked to find locations and answer questions about persons and objects encountered within the maze. This behavioral task was used to test patients that had received unilateral lesions of the left or right parahippocampal cortex to treat epilepsy. Those individuals that had received a right temporal lesion had difficulty in navigating the maze, whereas individuals that

received a left temporal lesion had greater difficulty in answering questions regarding persons and objects encountered within the maze.

Criticisms can be raised against the study of lateralization in epileptic patients as it could be argued that the patient's history of epilepsy may have influenced functional lateralization of their hippocampal formation. To address such concerns, further studies have been conducted to examine the functional lateralization of the human hippocampal formation in normal humans using more modern imaging techniques, including functional magnetic resonance imaging (fMRI) and magnetoencephalography (MEG) techniques (Tranel, 1991; Eldridge et al., 2000; Hanlon et al., 2005; Sommer et al., 2005; Ulrich et al., 2010). Many of these studies have focused on lateralization of the hippocampal formation during recall in language specific tasks (Tranel, 1991; Kelley et al., 1998; Eldridge et al., 2000; Ulrich et al., 2010); however some have focused specifically on recall in spatial memory tasks (Hanlon et al., 2005; Sommer et al., 2005).

Tranel (1991) found that an individual patient with damage to the left entorhinal cortex and hippocampal formation had deficits in the retrieval and acquisition of new verbal memories, whereas he had no deficits in the retrieval of non-verbal memories. Eldridge and colleagues (2000) used fMRI during a task where subjects were asked to memorize each word in a list and 20 minutes later the subjects were shown individual words that were either on the original list or were new words and then asked if the word was on the original list or novel and how confident they were of their answers. When the subject was confident that the word presented was on the original studied list, the subject had increased activity in the hippocampal formation. Additionally, activity was reduced in the hippocampal formation when the words were not present in the original list even

when the subject incorrectly identified the word as being on the original list. In comparing the responses in the left and right hippocampal formation Eldridge and colleagues also found that the changes in activity in the left hippocampal formation were greater than the changes observed in the right; thus, activity in the hippocampal formation was lateralized during recall.

Using fMRI, Ulrich and colleagues (2010) found that activity in the left hippocampal formation increased with increasing difficulty in an episodic memory encoding task. In the task, individuals were shown pictures of faces unknown to the individual with names underneath, and they were later asked to identify the name of the individual when the face was shown. The task was made more difficult by increasing the number of faces shown with names underneath at any given time (from 2-4) and then testing the individual’s recall later. Similar to the findings of Eldridge and colleagues (2000), activity in the hippocampal formation would increase bilaterally during recall; however, as the task became more difficult, the test subject would increasingly rely on the left hippocampal formation (Ulrich et al., 2010).

Sommer and colleagues (2005) used fMRI to examine activity in the brain during the encoding process of a memory task that was not directly related to language and instead required the association of an object with a location, in which the individual was later required to remember either the object or location based on the opposite cue. They found that whether an individual was presented with an object or a location cue they had increased activity bilaterally in the parahippocampal cortex. However, individuals who during retrieval successfully chose a location associated with an object cue showed increased activity in the left parahippocampal cortex during the original encoding.

Interestingly, their findings indicate that lateralized activity of the hippocampal formation is functionally significant. An individual could only reasonably later recall information that was originally learned; thus, Sommer and colleague's data indicate that the left hippocampal formation is preferentially involved in associating an object with a location in a learning task that allows for recall later.

Interestingly, Strange and colleagues (1999) argued that functional hippocampal lateralization is related to novelty detection. Adult male and female subjects (average age 21.7) were presented with an artificial grammar learning task that had novel components introduced throughout the task. Using fMRI, Strange et al. found that the left hippocampal formation showed increased activation in response to novelty in a verbal memory task. When words were repeatedly presented throughout a task, activity in the left hippocampal formation was reduced resulting in bilateral activity in the hippocampal formation with familiarity.

The above findings have been criticized as many of these tasks, whether directly related to language or not, required the recall of items from a list (Burgess, 2002). For this reason, some investigators have utilized virtual reality mazes to assess hippocampal dependent learning and memory in healthy adults (Maguire et al., 1998; Burges et al., 2001; Burgess 2002). Using positron emission tomography (PET) Maguire and colleagues (1998) found that when humans explored a virtual reality environment that was basically a maze with minimal external cues, they did not show lateralized activity in the hippocampal formation. In contrast, when the virtual maze was constructed to more closely resemble the reality an individual might actually encounter, such as details found on city streets, then they showed increased activity in the right hippocampal formation.

Anatomical Lateralization of the Human Hippocampal Formation

Anatomical asymmetries have also been observed in the human hippocampal region (Simic et al., 2005). Simic et al. (2005) studied normal human subjects ranging from 23-60 years of age and estimated the total number of neurons in layer II of the entorhinal cortex using an optical fractionator and measured the surface area. Simic and colleagues found an increase in the surface area of layer II in the left entorhinal cortex without a corresponding increase in the total number of neurons as compared to the right entorhinal cortex. They further proposed the interesting argument that the asymmetric afferent input to the entorhinal cortex from Broca's area contributes to the lateralization of the hippocampal region and may in part account for the requirement of the left hippocampal formation in verbal memory described above (Simic et al., 2005; Tranel, 1991; Kelley et al., 1998; Eldridge et al., 2000; Ystad et al., 2009; Ulrich et al., 2010).

An earlier study conducted by Zaidel and colleagues (1997) examined the neuroanatomy of the hippocampus proper and surrounding areas of the hippocampal region. They studied 10 μm coronal sections of postmortem human brain tissue stained with cresyl violet to determine neuronal cell body size, shape, and orientation in the left and right hippocampal region (CA1-CA4, and the subiculum) and found that neuron cell bodies were significantly larger in the left CA2 of healthy adult humans. In contrast to cell body size, Goncalves-Pereira and colleagues (2006) examined total hippocampal volume in adult humans and found that the right hippocampal volume was greater in right-handed individuals that ranged in age from 19-52 years old.

Thompson and colleagues (2008) examined hippocampal asymmetry in human newborns. Similar to Goncalves-Pereira and colleagues (2006) findings in adults, they

found that preterm and full-term human infants had greater right hippocampal volume at birth. MRI analysis was utilized to scan 184 preterm (22-32 weeks gestation) and 32 full-term infants. Once the MRI scans were complete the hippocampus was outlined on each coronal section of the series of scans to determine the hippocampal boundaries in order to calculate hippocampal volume. Thus, Thompson and colleagues found that lateralization of hippocampal volume in the human is present at birth.

Neurochemical Lateralization of the Human Hippocampal Formation

Neurochemical lateralization in the adult human hippocampal formation is neurotransmitter specific. Interestingly, when neurotransmitters were shown to be lateralized they were greater in the left hippocampal formation. For example, Glick and colleagues (1982) studied postmortem human brains and found that glutamic acid decarboxylase (GAD), gamma-aminobutyric acid (GABA), and choline acetyltransferase (ChAT) levels were greater in the left hippocampus. In contrast, Fink and colleagues (2009) later utilized positron emission tomography (PET) analysis of the radioligand carbonyl-11C WAY-100635 and found that the 5HT-1A receptor is not lateralized in the human hippocampal formation of right-handed male and female subjects. Similarly, DeLisi and colleagues (1989) examined glucose levels using [^{18}F]2-deoxy-D-glucose and cerebral blood flow analysis and found that glutamate levels were not lateralized in adult humans (average 28.4 years old). Thus, GABA and acetylcholine levels are likely greater in the left hippocampus (Glick et al., 1982) whereas serotonin (Fink et al., 2009) and glutamate (DeLisi et al., 1989) do not appear to be lateralized in the hippocampal formation of humans.

Lateralization of the Rodent Hippocampal Formation

Criticisms have been raised against the study of learning and memory deficits following lesions of the medial temporal lobe in humans due to the difficulty in finding patients that have lesions localized to specific brain structures. For example, the study of parahippocampal lesions is much more common in the study of human patients (Scoville and Milner, 1957), rather than studies of more localized lesions to subregions of the hippocampal formation that can be obtained using rats. For this reason, studies of experimental animals, including the rat, are used to focus on the subregions of the hippocampal formation. In rodents, lateralization of the hippocampal formation has been studied using genetic (Moskal et al., 2006), anatomical (Diamond et al., 1982; Verstynen et al., 2001; Lister et al., 2006), neurochemical (Kristofikova et al., 2004), physiological (Kawakami et al., 2003; Wu et al., 2005), and behavioral techniques that indicate a functional lateralization of the hippocampal formation (Bernasconi-Guastalla et al., 1994; Poe et al., 2000).

Functional Lateralization of the Rodent Hippocampal Formation

Behavioral studies indicate that lateralization of the hippocampal formation is functionally significant in the rodent. For example, Poe and colleagues (2000) showed that performance in the radial eight-arm maze was differentially affected by unilateral inactivation of the hippocampal formation in aged rats. In the radial eight-arm maze, rats were placed in a maze with eight arms and allowed to explore four arms at random. They then were required to wait in the center of the maze before all eight arms were opened again, which allowed the animals to obtain the remaining food rewards. Errors were

counted as revisits to any of the eight arms. Rats were tested in the radial eight-arm maze prior, during, and after unilateral inactivation of either the left, or right, hippocampal formation with 6% tetracane. Importantly, in examining the difference in performance following either left or right hippocampal inactivation, Poe and colleagues found that inactivation of the left, but not the right, hippocampus resulted in an increased number of errors during the radial eight-arm maze task. These findings indicate that the left, but not the right, hippocampal formation is necessary for the completion of the radial eight arm maze in aged rats.

Functional lateralization of the hippocampal formation has not been observed only in aged rats. In adult rats, Klur and colleagues (2009) recently showed different functions of the adult left and right hippocampal formation in the Morris water-maze task (MWMT). In the MWMT, rats are required to use spatial cues to determine the location of a platform located just beneath the water surface inside of a tank in order to stop swimming. Over multiple trials, rats will eventually learn to swim directly to the hidden platform (Morris et al., 1981). Klur and colleagues subjected rats to left, right, or bilateral inactivation of the hippocampal formation using lidocane prior to acquisition, or learning, trials in the MWMT. Inactivation of the left hippocampal formation produced the same learning deficits in the MWMT as bilateral inactivation, whereas inactivation of the right hippocampal had no effect. Thus, inactivation of the left, but not the right, hippocampal formation prevented learning in the MWMT. In contrast, when rats were subjected to hippocampal inactivation prior to a probe trial (in which they were required to remember the spatial location of the platform 24 hours after the last acquisition trial), right hippocampal inactivation produced deficits similar to bilateral inactivation, whereas

inactivation of the left hippocampal formation had no effect. Thus, inactivation of the right, but not the left, hippocampal formation prevented the rats from remembering the correct location of the platform that they had previously learned. Importantly, these findings indicate that the left hippocampal formation is involved in learning a task, while the right hippocampus is likely involved in the retrieval of previously learned information.

Using the MWMT, Bernasconi-Guastalla and colleagues (1994) also demonstrated that reversal learning is lateralized. In contrast to acquisition or probe trials like those used by Klur et al. (2009), reversal learning ascertains whether an animal can learn the location of a new escape platform once it has already learned the location of a previous platform. Bernasconi-Guastalla and colleagues found that mice varied in their ability to learn the location of the new platform; for that reason, they divided the mice into poor and good performers. Upon further examination, they found that reversal learning was much faster in mice with larger left intrapyramidal and infrapyramidal mossy fiber projections to pyramidal neurons in region CA3 of the hippocampus as compared to those mice that were classified as poor performers. Thus, Bernasconi-Guastalla and colleagues showed a correlation between performance in the MWMT and specific anatomical asymmetries in the mouse hippocampal formation.

Interestingly, lateralization of the hippocampal formation is not only functionally significant in adult and aged rats, but is also influenced by early experience (Tang, 2001; Verstynen et al., 2001; Zou and Tang, 2001; Tang et al., 2008). Tang and colleagues (2008) exposed rats to a novel environment for three minutes per day over the first three postnatal weeks and then examined the effect of that early postnatal experience on short

and long term potentiation (STP and LTP) in CA1 of adult (7 month old) rats. To examine STP and LTP, Tang and colleagues recorded excitatory postsynaptic potentials (EPSPs) following stimulation of Schaffer collateral axons of CA3 pyramidal cells that synapse on pyramidal cells of CA1. They found that novelty preferentially increased the induction of LTP in the left CA1 and the induction and maintenance of LTP in the right CA1. Unfortunately, they did not compare the EPSPs in the left and right hemisphere in either the control or novel group (Tang et al., 2008). However, their data clearly indicated that early experience had an asymmetric effect on the maintenance of LTP in CA1 of the rat hippocampus.

Anatomical Lateralization of the Rodent Hippocampal Formation

Studies of anatomical lateralization of the rat hippocampal indicate that hippocampal volume is greater in the right hemisphere before P90 and is greater in the left hemisphere after P90 (Diamond et al., 1982; Verstynen et al., 2001; Lister et al., 2006). Diamond and colleagues (1982) found that rat hippocampal volume was 8% greater in the right hemisphere in young male rats and this asymmetry decreased until it was no longer observed at P90. However, Lister and colleagues (2006) later showed that the volume of the stratum pyramidale was 6% greater in the left CA3/CA2 than in the right and 21% greater in the left CA1 region in Sprague-Dawley rats; thus, the total number of neurons was likely greater in the left hippocampus at P90. Additionally, Lister et al. found that the width of the granule cell layer was not lateralized at P90. The cell layer volumes were also not lateralized at P90 in the presubiculum and parasubiculum. Thus, asymmetry of hippocampal volume was specific to the hippocampus at P90 and did not include the

dentate gyrus or the subiculum. Furthermore, taken together these findings indicate a right-to-left shift in hippocampal volume occurs around P90 in the rat hippocampus.

Importantly, Tang and colleagues (Verstynen et al., 2001) have shown that rat hippocampal volume is influenced be early postnatal experience. Handling and novelty exposure for 3 minutes per day over the first three postnatal weeks resulted in a loss of volumetric asymmetry. Results showed that 8 month-old adult rats that were not exposed to novelty over the first three postnatal weeks had a greater left hippocampal volume, whereas, following novelty exposure the volume of the left hippocampal formation decreased, resulting in a loss of hippocampal volumetric asymmetry. In considering Verstynen and colleague's results in combination with Diamond et al. (1982) and Lister et al. (2006), data indicate a right-to-left shift in hippocampal volume normally occurs, and that handling and novelty exposure may affect that shift, resulting in a loss of asymmetric hippocampal volume in the adult rat.

Neurochemical Lateralization of Rodent Hippocampal Formation

Kristofikova and colleagues (2004) have noted changes in the lateralization of high affinity choline uptake (HACU) over the lifespan of male rats. Total HACU increased between P7 and P60 bilaterally, began to drop at six months of age and dropped significantly in aged rats. In more closely examining HACU in the rat hippocampal formation, they found that HACU was clearly lateralized in young adult male rats: HACU was greater in the left hippocampal formation of 2.5-3.5 month-old male rats.

The lateralization of HACU in the rat hippocampal formation of adult male rats was due to an increase in HACU in the left hippocampal formation during maturation. HACU

was not significantly different between the left and right hippocampal formation until male rats reached adulthood; however, the unilateral increase in HACU in the left hippocampal formation during development was significant at P14 when compared to P7 (Kristofikova et al., 2004). By 2.5-3.5 months of age HACU was significantly greater in the left hippocampal formation as compared to the right. Thus, HACU increased preferentially in the left rat hippocampal formation during early development.

In addition to studying the lateralization of HACU during development, Kristofikova and colleagues (2004) also studied the role of NMDAR-mediated synaptic activity on lateralization of HACU. Intracerebroventricular administration of quinolinic acid, an NMDAR agonist, at P12 resulted in a loss of asymmetric HACU in 2 month old rats. Interestingly, quinolinic acid administration at P12 resulted in a more pronounced reduction in HACU in the left hippocampal formation as compared to the right. Thus, an NMDAR agonist administered prior to the end of second postnatal week resulted in a loss of the asymmetric HACU that was normally observed in adult rats.

Previous findings further suggest that the ε2 subunit (NR2B subunit in the rat) of the NMDAR is differentially expressed at particular synapses in CA1 of the adult mouse hippocampus (Kawakami et al., 2003; Wu et al., 2005; Shinohara et al., 2008). The NMDA glutamate receptor ε2 subunit was shown to be lateralized in hippocampal CA1 pyramidal neurons in mice: the ε2 subunit density was greatest in the left apical and right basal dendrites (Kawakami et al., 2003). Kawakami and colleagues stimulated Schaffer collaterals in stratum radiatum or stratum oriens of CA1 and recorded excitatory post synaptic currents (EPSCs) of CA1 pyramidal cells using whole cell recordings in mice with a transected ventral hippocampal commissure. Using a pharmacological blocker of

the ε2 subunit of the NMDAR (Ro 25-6981), they noted that the ε2 subunit had a greater contribution to the total EPSC when Schaffer collaterals were stimulated in the left stratum radiatum or the right stratum oriens.

Interestingly, Wu et al. (2005) showed that lateralization of the ε2 subunit was not observed in interneurons of CA1 ε1 knock-out mice, suggesting that lateralization of at least the NMDARε2 subunit may be cell type specific. Interneurons in CA1 of the rat hippocampus receive afferent input from Schaffer collaterals of CA3 pyramidal neurons (see Chapter 2). The density of NMDARε2 subunit was not significantly different between the left and right Schaffer collateral to CA1 interneuron synapses in ε1 knock-out mice. Thus, CA1 interneurons likely receive equal afferent input from the left and right CA3 Schaffers. These findings led Wu and colleagues to suggest that the regulation of the differential expression of the ε2 subunit of the NMDAR is likely dependent on the type of presynaptic neuron.

In contrast to Wu and colleagues, Shinohara and colleagues (2008) argued that the differential distribution of NMDARε2 subunits in the CA1 region of the rat hippocampus is dependent upon presynaptic Schaffer collateral input from the CA3 region. They further investigated the differential distribution of the ε2 subunit of the NMDAR in CA1 and found that the ε2 subunit was differentially distributed in CA1 pyramidal cell dendritic spines. Interestingly, they found that mushroom spines on CA1 pyramidal cell dendrites in stratum radiatum preferentially receive afferent input from the right CA3 Schaffer collaterals, whereas thin spines preferentially receive afferent input from left CA3 Schaffers. Additionally, the small thin spines have a greater number of NMDARε2 subunits, whereas the large mushroom spines have a greater number of GluR1 receptors

resulting in a spine with a low ε2 subunit density. Thus, the asymmetric distribution of glutamate receptors in CA1 pyramidal cells was shown to be correlated with the presynaptic Schaffer collateral axons from CA3.

Molecular Lateralization of the Rodent Hippocampal Formation

Lateralized gene expression (Moskal et al., 2006; Klur et al., 2009) and protein levels (Samara et al., 2011) have been observed in the rat hippocampal formation. Our lab utilized microarray analysis to examine lateralized gene expression at the end of the first postnatal week (P6 and P9) and in young adult rats (P60; Moskal et al., 2006). Of the genes that were differentially expressed, all were more highly expressed in the right hippocampal formation at P6, and the majority of the differentially expressed genes were more highly expressed in the left hippocampal formation at P9 and P60. Thus, a right-to-left shift in lateralized gene expression occurred between P6 and P9. Furthermore, these data clearly indicate that lateralized gene expression is observed as early as P6 in the rat hippocampal formation and is still observed in the adult rat. Of the genes differentially expressed during hippocampal development, many correspond to proteins involved in synaptic function, cellular morphology, and vesicle trafficking (Moskal et al., 2006).

Klur and colleagues (2009) later examined ipsilateral gene expression in CA1 of the rat hippocampus in adult rats (2-3 months old) either tested in the MWMT or control rats that were allowed to swim in the tank in a situation in which the platform was visible and, thus, the rat could escape without having to learn where the platform was located. Using microarray analyses, Klur and colleagues (2009) observed changes in both the left and right CA1 following learning as compared to the left and right CA1 of the control rats. In

the right CA1 region, the expression of 56 genes changed following learning: 35 genes were more highly expressed following learning and 17 genes were repressed following learning. Of the 56 genes, many corresponded to proteins involved in cellular plasticity and learning and memory, including proteins related to gene transcription, protein synthesis and degradation, cell structure and growth, cell signaling and vesicular transport. In the left CA1 region, 20 genes were more highly expressed following learning. Of those 20, many also coded for proteins involved in cellular cell structure and growth, DNA transcription and protein synthesis, and cellular signaling and transport (Klur and colleagues 2009).

Samara and colleagues (2011) later determined lateralized protein levels in the rat hippocampus of young adult male rats, and they found that 80 proteins were lateralized. Of the proteins that were at higher levels in the right hippocampus, many were involved in cellular metabolism. In contrast, the proteins at higher levels in the left hippocampus were typically found in astrocytes. Thus, Samara and colleagues (2011) observed lateralized protein levels in the rat hippocampal formation that may be functionally significant.

In an effort to understand the molecular mechanisms that underlie the development of hippocampal asymmetry in the mouse, Kawakami and colleagues (2008) measured the differential expression of the ε2 subunit of the NMDAR in *iv* mice. Individuals that lack the *iv* gene have either *situs inversus* (complete reversal of directional asymmetry in the body – most notably that organ asymmetry is reversed), or *situs solitus* (correct directional asymmetry in the body); thus, a loss of *iv* expression leads to a loss of directional asymmetry in the body. For this reason, it has been suggested that the inversus

viscerum (*iv)* gene has an important role in the establishment of asymmetry (Hamada et al., 2002). As mentioned above, Kawakami and colleagues (2003) have previously shown that the ε2 subunit of the NMDAR is more highly expressed in the left stratum radiatum of CA1. Mice that no longer express the *iv* gene (whether those with *situs inversus* or *situs solitus*) show a loss of differential expression of the ε2 subunit of the NMDAR (Kawakami et al., 2008). These findings indicate that at least some directional asymmetries are lost within the brain in *iv* mice. Thus, the molecular mechanisms that establish brain asymmetry may be related to those that establish peripheral asymmetry.

Conclusions

In the present study, I utilized rats to examine the development of hippocampal lateralization. Rats were chosen because they have a hippocampal formation that is morphologically and functionally similar to humans (see Chapter 2) and the majority of prior research on cortical lateralization, in species other than humans, has been obtained using rats (see Chapter 3). Furthermore, the findings summarized in the present chapter indicate that hippocampal lateralization in rats and humans is markedly similar when considering functional lateralization. The left hippocampal formation is necessary for associational learning, whereas the right is necessary for recall. In contrast, potentially significant differences between humans and rats are observed in volumetric asymmetry.

Unilateral lesions, or unilateral inactivation, of the hippocampal formation are sufficient to produce learning and memory deficits in humans (Abrahams et al., 1997; Bohbot et al., 1998; Spiers et al., 2001; Binder et al., 2009; Glickman-Johnton et al., 2008; Mechanic-Hamilton et al., 2009; Barkas et al., 2010; Bonelli et al., 2010) and

rodents (Poe et al., 2000; Klur et al., 2009). Sommer and colleagues (2005) found that humans preferentially use the left hippocampal formation during learning in a task that required the specific association of an image on a cue card with the original location in a series. In adult rats, Klur and colleagues (2009) later showed that inactivation of the left hippocampal formation inhibited learning in the MWMT (Klur et al., 2009). Thus, both humans and rats preferentially rely on the left hippocampal formation to learn tasks that require the specific association of a cue (either the original image in a series, or the external cues in the MWMT) with the correct response (either the correct location in the series, or the escape platform in the MWMT). Taken together, these findings indicate that both humans and rats require the left hippocampal formation for associational learning.

In contrast to the associational learning tasks described above, Spiers et al (2001) examined performance in a maze where an individual was required to answer questions about what they had encountered within the maze. Importantly, these individuals were simply asked about their recall of facts, rather than associating a cue with the completion of the task. They found that individuals with a left temporal lesion had greater difficulty in answering questions regarding persons and objects encountered within a virtual maze. Similarly, Ulrich et al. (2010) found that humans increasingly rely on the left hippocampal formation when the recall task is difficult. Comparable to findings in humans (Spiers et al., 2001a; 2001b; Ulrich et al. 2010), Poe et al (2000) studied rat performance in a behavioral task that required memorizing a particular sequence, rather than the use of associational memory. More specifically, Poe and colleagues (2000) scrutinized performance in the radial eight-arm maze where rats were required to remember which 4 arms were previously visited in order to obtain a food reward in the

remaining 4 arms after being allowed access to all eight arms. In contrast to experiments of Klur and colleagues (2009) described above, the radial eight-arm maze sequence changed with each trial; therefore, instead of memorizing a particular sequence, the rats were required to apply a general rule (enter previously unvisited arms to obtain a food reward) to successfully complete the task. They found that inactivation of the left hippocampal formation resulted in increased errors in the radial-eight-arm-maze in aged rats (Poe et al., 2000). Taken together, these findings indicate that the left hippocampal formation is also required to memorize a sequence of information.

The hippocampal formation has also been shown to be involved in recall in humans (Scoville and Milner, 1957; Langston and Wood, 2008; Lehn et al., 2009) and rats (Butterly, 2011; reviewed in Rolls and Kesner, 2006). In humans, it has been suggested that the right hippocampal formation is involved in the recall of information (Smith and Milner, 1981; Nunn et al., 1999; Jones-Gotman, 1986). To assess the role of hippocampal lateralization in recall in rats, Klur and colleagues (2009) very clearly studied both learning and recall in the MWMT. To accomplish this goal, they utilized a probe trial. In the experimental design the rats were originally tested in acquisition trials where they learned to associate the external cues of the maze with the location of the escape platform. After the acquisition trials, the rats were then tested in a probe trial 1 day after the last acquisition trial to assess whether the rat recalled the location of the escape platform. They found that while the left hemisphere was required to learn in the acquisition trials of the MWMT, the right hemisphere was specifically required to recall the previously learned information in the MWMT during the probe trial. Thus, in both

humans and rats, the right hippocampal formation has been shown to be necessary for recall.

In contrast to the above studies indicating that the left hippocampal formation is preferentially involved in hippocampal dependent learning, others have proposed that the right hippocampal formation, and not the left, is necessary for learning (Bohbot et al., 1998; Spiers et al., 2001). They argued that deficits in navigating a task similar to the MWMT (Bohbot et al., 1998) or a computer maze (Spiers et al., 2001) were observed following lesions of the right, but not the left, parahippocampal region in humans. One possible explanation for these discrepancies is that the tests did not adequate assess learning. For example, Bohbot and colleagues had the individuals locate a hidden sensor in a room with spatial cues similar to the MWMT. In instances where a 30 minute delay was added to the task, individuals with lesions the right parahippocampal cortex were less successful at locating the hidden sensor. The incorporation of a delay prior to repeating the task rather than the use of repeated acquisition trials would make Bohbot and colleagues (1998) experiment more similar to a probe trial; therefore, these findings do not preclude the possibility that the left hippocampal formation is preferentially involved in learning. Additionally, Spiers and colleagues (2001) had the individual answer questions regarding specific information from characters encountered while navigating a virtual maze: this particular task would more likely involve recall of information, rather than simply assessing spatial learning. Thus, these findings do not in fact contradict those observed following left hippocampal lesions in humans.

Behavioral lateralization of the rat hippocampal formation has been shown to be correlated with anatomical asymmetries in rats, where rats with larger left infrapyramidal

mossy-fiber axons performed better on the MWMT (Bernasconi-Guastalla et al., 1994). The left hippocampal formation has also been shown to be larger in adult rats (Verstynen et al., 2001) and the left entorhinal cortex has been shown to be larger in adult humans (Simic et al., 2005). Thus, in both adult humans and rats the left hippocampal volume is greater.

Neurochemical lateralization of the hippocampal formation has been more broadly studied in humans. Glick and colleagues (1982) found that GABA and acetylcholine levels were greater in the left human hippocampus. Similar to these findings, Kristofikova et al., (2004) found that high affinity choline uptake increased preferentially in the left hippocampus during early development and was greater in the left hippocampus of adult rats. DeLisi and colleagues (1989) found that glutamate levels were not asymmetric in adult humans. Although lateralized glutamate levels have not been examined in the rat hippocampal formation, Kawakami et al. (2003) found that the ε2 subunit of the NMDAR was differentially distributed in the CA1 region of the rat hippocampus.

Importantly, the study of hippocampal lateralization using animal models allows for the closer examination of molecular lateralization without having to solely rely on the study of postmortem human brains. Using microarray techniques, our lab was able to show that lateralized gene expression was present in during early postnatal development in the rat and in the young adult rat (Moskal et al., 2006). Learning has been shown to change lateralized gene expression in the adult rat (Klur et al., 2009). Although our previous findings indicate that lateralized gene expression is observed during early postnatal development (Moskal et al., 2006), it has yet to be determined whether

lateralization is observed at earlier points in development. The objective of my dissertation is to characterize hippocampal lateralization during development in the rat.

Denenberg (Denenberg et al. 1981; Denenberg, 2005) argued that lateralization only emerges as a result of experience during postnatal development in the rat. In contrast, Sun and colleagues (2005, 2006) have observed lateralized gene expression during embryonic development of the human cortex. These contrasting findings lead to two important questions related to the development of the brain: when is lateralization first established and how is it influenced by experience during early postnatal development? My hypothesis is that hippocampal lateralization will be observed during embryonic development and that it will be influenced by a reduction in N-methyl-D-aspartate glutamate receptor (NMDAR) mediated synaptic activity in the rat.

CHAPTER 5. LATERALIZED GENE EXPRESSION IN THE RAT HIPPOCAMPAL FORMATION AT EMBRYONIC DAY 18

INTRODUCTION

In humans and in rodents, many brain regions, including the hippocampal formation, show differences between the left and right hemispheres (deToledo-Morrell et al., 1988; Bernasconi-Guastalla et al., 1994; Tabibnia et al., 1999; Milner, 1998; Poe et al., 2000; Spiers et al., 2001; Hanlon et al., 2005; Sommer et al., 2005; Lister et al., 2006; Moskal et al., 2006; Thompson et al., 2008; Klur et al., 2009). The hippocampal formation is necessary for some forms of learning and memory (Olton and Samuelson, 1976; Morris et al., 1982), and interestingly, lateralization of the hippocampal formation has been shown to be functionally significant in adults (deToledo-Morrell et al., 1988; Bernasconi-Guastalla et al., 1994; Milner, 1998; Poe et al., 2000; Spiers et al., 2001; Hanlon et al., 2005; Sommer et al., 2005; Klur et al., 2009). Furthermore, volumetric differences in lateralization of the hippocampal formation as compared to normal age-matched controls have been observed in neuropsychiatric disorders, such as autism (Schuman et al., 2004; Nicolson et al., 2006) and schizophrenia (Crow and Harrington, 1994; Harrison, 1999; Zaidel et al., 1999; Spaniel et al., 2003; Hanlon et al., 2005; Hanlon and Sutherland, 2005). These differences in hippocampal lateralization are likely established during early development. Thus, it is important to characterize the development of hippocampal lateralization in order to more fully understand when changes in lateralization might contribute to the development of neuropsychiatric disorders. However, very few studies have been directed toward understanding the development of hippocampal asymmetry.

The rat hippocampal formation begins to develop at E15, when neurogenesis is first observed in the hippocampus proper (Schlessinger et al., 1975; Bayer et al., 1980a). However, the primary focus of studies on the development of hippocampal lateralization have been on the effect of environmental changes during early postnatal development on functional lateralization of the adult hippocampal formation (Verstynen et al., 2001; Tang et al., 2008). For example, novelty exposure for 3 minutes per day over the first three postnatal weeks resulted in a loss of volumetric hippocampal asymmetry (Verstynen et al., 2001) and increased maintenance of long-term potentiation in the right CA1 following stimulation of Shaffer collateral axons in stratum-radiatum of 7-8 month old adult male rats (Tang et al., 2008). Verstynen and colleagues (2001) found that adult rats not exposed to novelty over the first three postnatal weeks had greater left hippocampal volume. In contrast, following novelty exposure during early development, the volume of the left hippocampal formation decreased resulting in a loss of hippocampal volumetric asymmetry as compared to normal rats. In order to determine whether novelty exposure during early development also influenced hippocampal physiology, Tang and colleagues (2008) further examined the effect of novelty exposure during early development on short and long term potentiation (STP and LTP) in CA1 of adult rats. To examine STP and LTP they recorded excitatory postsynaptic potentials (EPSPs) in CA1 pyramidal neurons following stimulation of Schaffer collateral axons of CA3 pyramidal neurons cells. They found that novelty preferentially increased the induction and maintenance of LTP in the right CA1. Unfortunately, they only made unilateral comparisons in the control and novel group rather than comparing the left and right hemisphere in each group (Tang et al., 2008). However, their data clearly indicated that early experience had an asymmetric

effect on the maintenance of LTP in CA1 of the rat hippocampus. These previous findings from Tang and colleagues (Verstynen et al., 2001; Tang et al., 2008) indicate that adult hippocampal lateralization is influenced by early postnatal experience and, yet, these studies do not indicate whether lateralization is observed during embryonic development.

The only previous indication that lateralization of the hippocampal formation is present during development was a study of volumetric asymmetry in human infants (Thompson et al., 2008) and work conducted in our lab indicating that genes are differentially expressed in the hippocampal formation during early postnatal development in the rat (Moskal et al., 2006). Thompson and colleagues (2008) found that both preterm and full-term human infants had greater right hippocampal volume at birth. A previous study in our lab indicated that lateralized gene expression was observed as early as P6 in the rat (Moskal et al., 2006). This study showed that all of the differentially-expressed genes were more highly expressed in the right hippocampus. Surprisingly, it has yet to be determined whether hippocampal lateralization is observed at earlier points in development in the rat. Thus, an important question in the study of hippocampal development remains: when is hippocampal lateralization established?

Tang (2008) has suggested that hippocampal lateralization is established prior to birth and is modified by experience over the lifespan. The only indication that lateralization of the brain is observed prior to birth was a recent study conducted by Sun and colleagues (2006) where lateralized gene expression was observed in the perisylvian cortex of the human embryo. They examined gene expression in the cortex at weeks 12 and 14 of human embryonic development. They found that the majority of differentially-expressed

genes in the cortex corresponded to proteins that regulate cell development, signaling, communication, metabolism, and gene or protein expression. These data suggest that at least some forms of lateralization are established during embryonic development in the human. However, it has yet to be determined whether hippocampal lateralization is observed during embryonic development in either the human or the rodent. The objectives of the present study were to determine whether hippocampal lateralization is observed during embryonic development of the rat, and, if so, to more closely examine the pattern of lateralized gene expression to determine any relationship between lateralized gene expression and the development of the hippocampal formation.

In the present study, microarray and qRT-PCR analyses were used to examine the pattern of lateralized gene expression in the embryonic rat hippocampus. As I noted above, the hippocampal formation begins to develop at E15; however, in the present study, gene expression was examined at E18 because the hippocampus can be easily visualized and the principal neurons of the hippocampal formation are born by this age (Schlessinger et al., 1978; Bayer, 1980). Additionally, western blot analysis was used to examine lateralized protein levels in the hippocampus at E18. Results indicated that genes were more highly expressed in the right hippocampus at E18.

METHODS

Animals: Timed-pregnant Sprague-Dawley rats (Charles River Labs, Wilmington, MA) and their pups at E18 were used in this study. Rats were housed either in the University of Texas at San Antonio (UTSA) or the University of New Mexico (UNM) Animal Facilities and provided with food and water *ad libitum* and kept on a 12 hour light dark

cycle. All procedures were approved by the UTSA and UNM Institutional Animal Care and Use Committees and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Hippocampal Dissections: Timed-pregnant female rats at E18 (n = 10) were anesthetized with 4% isoflurane (Halocarbon Products, River Edge, NJ) and oxygen at 2 liters per minute using an Ohio style vaporizer (Model 100H, Surgivet/Anesco, Waukesha, WI) until a vigorous tail pinch no longer elicited a response. The uterus was removed and placed in a sterile Petri dish with ice-cold saline and the female was decapitated under deep general anesthesia after again ensuring that a tail pinch did not elicit a response. Embryos at E18 were chosen because the hippocampus can be easily visualized at this age (Schlessinger et al., 1978; Bayer, 1980a). The left and right hippocampi of the E18 rat pups were dissected and used for either gene expression or protein level assays as described below.

For the gene expression assays, after the embryos were decapitated under deep general anesthesia the entire head was placed in RNA*later* (Ambion, Inc., Austin, TX) at 4˚C to immediately reduce RNAse activity (Mutter et al., 2004; Vincent and Deutscher, 2009). To dissect the E18 hippocampi, the meninges and choroid plexus were removed with fine forceps, and the hemispheres were separated. RNA*later* was added again once the brain was completely removed and the hemispheres were separated. Each hippocampus was removed from the adjoining cortex by a cut parallel to the hippocampal fissure and transverse cuts at the rostral and caudal ends while observing the tissue under a dissecting microscope (Banker and Cowan, 1977; Banker and Goslin, 1998). Once the left and right hippocampi were dissected, they were placed in separate 1 ml aliquots of

RNA*later*. The remaining brain tissue was also stored in RNA*later* in order to genotype the E18 rat pups to determine the sex.

To determine protein levels, the uterus was removed and placed in ice-cold saline; embryos were removed and then decapitated. The brain was removed and immediately frozen on dry ice and stored at -80˚ C. Prior to isolating protein from either the left or right hippocampus at E18 the hippocampi were dissected while the brain tissue remained frozen (Banker and Cowan, 1976; Kroes et al., 2006). A small block of tissue that included the hippocampus was dissected using a razor blade by making coronal cuts anterior and posterior to the embryonic hippocampus. The hippocampus was then dissected under a dissecting microscope (Banker and Cowan, 1976; Kroes et al., 2006). Once the hippocampus was removed, the protein was immediately isolated as described below. The remaining brain tissue was used to genotype the E18 rat pups.

Genotyping: In order to isolate genomic DNA, I used a modified protocol from Laird et al. (1991). In an effort to isolate a sufficient amount of genomic DNA from such small tissue samples and to expedite the process of isolating genomic DNA from so many individual samples, I used a tissue homogenizer to disrupt cells and extract DNA, rather than a mortar and pestle. E18 rat pups (n = 62) were genotyped by examining the presence of the sex-region Y (SRY) gene (An et. al., 1997). Additionally, it is important to note that I tested the modification of the protocol from Laird et al. (1991) and the primers utilized by An et al. (1997) on adult male and female brain tissue to ensure the procedure could be used to correctly identify male rats at E18 (data not shown).

Brain tissue previously stored in RNAlater was homogenized in DNA lysis buffer with SDS (100mM Tris · HCL pH 8.5, 5mM EDTA, 0.2% SDS, 200 mM NaCl) to

solubilize membrane lipids (Laird et al., 1991). Proteinase K (1µg/µl) was then added to digest proteins. To extract the DNA, 2.0 ml of tris buffered phenol (pH 8.0), chloroform, and isoamyl alcohol (PCI: 25:24:1) solution was added to the samples and they were gently inverted for 5 minutes and then centrifuged at 2000 rpm for 5 min. The PCI extraction was repeated on the aqueous layer. Next, a solution of 2.0 ml of chloroform and isoamylalcohol (CI: 24:1) was used to further extract genomic DNA. An equal volume of 7.5M ammonium acetate and 2.5 ml of 100% ethanol was added to the aqueous layer. Genomic DNA was then collected using a glass rod and briefly washed in 70% ethanol. The DNA was dissolved in 400 µl of dH_2O. RNA was then removed with 1mg/ml RNaseA following incubation at 37˚C for 30 minutes. PCI (25:24:1) was added and the PCI extraction was repeated. To suspend the DNA, 40µl of 3M sodium acetate and 100µl 100% ethanol was added to the aqueous phase. The DNA was again collected with a glass rod and resuspended in 400µl TE (pH 8.0). DNA was quantified using a nanodrop spectrophotometer by determining the absorbance at 260nm and then stored at 4˚C. Once the genomic DNA was isolated, the rats were genotyped using the SRY gene (An et al., 1997). The SRY primer sequences used were: *5'* primer, 5'-CATCGAAGGGTTAAAGTGCCA-3' and 3' primer, 5'-ATAGTGTGTAGGTl'GTTGTCC-3' in the following PCR conditions: thirty amplification cycles; denaturation step at 94˚C for 1.25 minutes, an annealing step at 58˚C for 2.5 minutes, and an extension step at 72˚C for 2.5 minutes (An et al., 1997; data not shown).

RNA Isolation: RNA from individual male rats at E18 was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA) adapted from the single-step RNA

isolation method developed by Chomczynski and Sacchi (1987) that utilizes guanidinium-thiocyanate. This method allows for the purification of RNA from lipid-rich tissues, such as those found in the brain (Chominczynski and Sacchi, 1987) based on the properties of a silica-based membrane that contains a high-salt buffer allowing up to 100 μg of total RNA to bind to the membrane. The left and right hippocampal tissue samples were homogenized in acidic guanidinium-thiocyanate phenol buffer solution (QIAzol Lysis Reagent; Qiagen Valencia, CA) that inhibits RNase activity and dissociates nucleoprotein complexes (Damodaran and Kinsella 1983; Chominczynski and Sacchi, 1987). Chloroform was utilized to allow for phase separation following centrifugation at 4˚C: the acidic aqueous phase contained mostly RNA, rather than DNA or proteins (Chominczynski and Sacchi, 1987). Ethanol was added to the aqueous phase to precipitate the RNA (Shapiro 1981; Chominczynski and Sacchi, 1987) and the solution was added to the silica membrane column that contained the total RNA from the hippocampal tissue sample. The column was washed with RWI buffer (Qiagen, Valencia, CA) to remove any QIAzol Lysis Reagent contaminants in the RNA sample. DNase I was added to the column for 15 minutes to ensure that no DNA remained in the isolated sample. The column was washed with RPE buffer (Qiagen, Valencia, CA) and allowed to dry in order to remove any traces of ethanol. Total RNA was eluted in 30 μl of RNAse-free water and then eluted again using the first elute and quantified using a NanoDrop spectrophotometer (Thermoscientific, Wilmington, DE) by determining the absorbance at 260 nm. The final RNA samples were stored at -80˚C for future use. To ensure that dissection order did not influence RNA yield and possible lateralized gene expression patterns, I alternated which hemisphere was dissected first: dissection order and the

hemisphere dissected had no effect on RNA yield or RNA quality as determined by the 260/280 and 260/230 ratios (data not shown).

Protein Isolation: Total protein was isolated and quantified from frozen E18 hippocampal tissue using the BCA assay developed by Smith and colleagues (1985). Briefly, lysis buffer containing 62.5 mM Tris (pH 6.8), 2% SDS, 5% glycerol, 10 mM sodium orthovanadate, 1% β-mercaptoethanol, and protease inhibitors was heated to 90˚C for 10 minutes prior to being added to isolated frozen hippocampal tissue. The tissue in lysis buffer was then homogenized using a dounce homogenizer. The homogenized samples were boiled at 90˚C. Proteins were quantified by determining the absorbance at 562nm against a standard curve of bovine serum albumin (BSA) protein levels following incubation of the samples at 37˚C with reagent A (1% BCA-Na_2, 2% $Na_2CO_3 \cdot H_2O$, 0.16% Na_2 tartrate, 0.4% NaOH, and 0.95% $NaHCO_3$, pH: 11.25) and reagent B (4% $CuSO_4 \cdot 5H_2O$) for 30 min (Smith et al., 1985; Thermo Scientific, Wilmington, DE; data not shown).

Microarray Analysis: I used an array designed by Kroes and colleagues (2006) to assay the expression of 1,178 genes specific to the rat brain and representing more than 90% of the major gene ontological categories in the left and right hippocampi of E18 rats. The rat CNS microarray was designed with 45 base pair sequence oligonucleotides complimentary for the 1,178 messenger RNAs (mRNAs) based on the following criteria: 1) minimal secondary structure, 2) minimal homology to other genes in available databases, such as NCBI/EMBL/TIGR, 3) no repeat regions, and 4) defined Tm using ArrayDesigner software (Lockhart et al., 1996; Kroes et al., 2006). Oligonucleotides were then synthesized using a PolyPlex 96 well oligonucleotide synthesizer (GeneMachines,

San Carlos, CA) using phosphoramidite chemistry and printed using an OmniGrid microarrayer (GeneMachines, San Carlos, CA) on an aldehyde glass microscope slide 250μm apart. The accuracy, reproducibility, and specificity of the microarray was evaluated previously (Kroes et al., 2006) using mRNA spiking experiments (Baum et al., 2003).

Total RNA isolated was pooled by hemisphere and by litter from individual E18 male left or right hippocampi and then were amplified and labeled (Van Gelder et al., 1990; n = 20 male rats at E18 from 5 litters). Specifically, reverse transcription of RNA with an oligo(dT) primer with a T7 promoter was followed by *in vitro* transcription in the presence of amino-allyl dUTP. The RNA was pooled from either the left or right hippocampus of male E18 rats in each litter for a total of five litters: 7 males from the first litter, 2 from the second litter, 4 from the third and fourth litter, and 3 from the fifth litter. Three microarray slides were used for each pooled sample, and each oligonucleotide was spotted in quadruplicate on each slide; thus, with 5 pooled litters there were 60 expression measurements for each gene in either the left or right E18 hippocampus. I utilized universal rat reference RNA (Stratagene) in my analyses and treated those aliquots concurrently with the tissue samples. Experimental (10μg of either left or right hippocampal aRNA labeled with Cy5) and reference (10 μg labeled with Cy3) amplified RNA were combined and hybridized to the microarray slide for 16 hours at 46˚C. Following washes, Cy3 and Cy5 fluorescence hybridization to each spot on the microarray was quantified using a high-resolution confocal laser scanner (Kroes et al., 2006).

After scanning, I utilized Significant Analysis of Microarrays (SAM) to determine the number of differentially expressed genes with a false discovery rate of less than 10% (Tusher et al., 2001; van de Wiel, 2004; available at http://www-stat.stanford.edu/~tibs/SAM/). I used SAM analysis because, importantly, if standard t-tests with a p-value cutoff of 0.01 were utilized on the 1,178 genes assayed this would identify 11 genes by chance (Tusher et al., 2001; Kroes et al., 2006): to solve this problem SAM is used to determine a false discovery rate score based on a gene-specific t-test of 500 random permutations (Tusher et al., 2001). Fold changes were determined by looking at the observed versus expected relative difference scores, or the ratio of the change in gene expression to the standard deviation. If gene expression was not lateralized, the majority of the 1,178 genes would be expected to have equal expression in the left and right hippocampus at E18: in that case the observed versus expected relative difference scores would be equal (Tusher et al., 2001; Kroes et al., 2006). However, some of the genes observed were sufficiently different from the expected relative difference score, indicating that those genes were in fact differentially expressed. The fold change values indicate the level of differential gene expression in the E18 hippocampus. For example, a fold change of 1.0 would indicate no difference in gene expression between the left and right hippocampi, whereas a fold change of 1.10 would indicate a 10% increase in gene expression in the right hippocampus as compared to the left. Importantly, fold change values are independent of the false discovery rate calculation. The false discovery rate is the percentage of genes that would be false positives, or shown to be differentially expressed by chance (Tusher et al., 2001).

Database for Annotation Visualization and Integrated Discovery (DAVID) Analysis of Microarray: Importantly, while the 1,178 genes assayed do not represent the complete rat genome they do represent more than 90% of the major gene ontological categories (Kroes et al., 2006), thereby allowing us to gain insights into biological mechanisms utilizing ontological analyses. Ontological analysis allows for the identification of additional genes not included in the original microarray that are important to cellular pathways. For this reason, I chose to more closely examine the role of the genes that were shown to be differentially expressed in the hippocampus for their role in specific gene ontology categories utilizing DAVID (Dennis et al., 2003; Huang et al., 2009; available at http://david.abcc.ncifcrf.gov/), GOMiner (Zeeburg et al., 2005; available at http://discover.nc.nih.gov/gominer), and GSEA analysis (Subramanian et al., 2005; Subramanian et al., 2007; available at http://www.broadinstitute.org/gsea/).

I utilized DAVID gene functional classification and gene functional annotation tables to examine interrelated genes within the gene list obtained using SAM analysis. DAVID functional classification tables with medium classification stringency were generated to examine genes within the gene set based on functional similarity (Huang et al., 2009). Additionally, I used DAVID Functional Annotation Analysis to examine molecular function, cellular components, and biological process gene ontology categories and molecular pathways.

GoMINER Analysis: GoMiner (Zeeburg et al., 2003; Zeeburg et al., 2005; available at http://discover.nc.nih.gov/gominer) uses gene ontology (GO) categories to organize genes based on biological processes, molecular function, or subcellular localization. Results indicate which ontological categories are depleted or enriched (Zeeburg et al.,

2003). The total set of genes on the array and the subset of genes that were identified as significantly differentially expressed using SAM analysis were further analyzed using GoMiner. The enrichment of any gene ontology category within the differentially expressed gene set obtained using SAM was determined using the two-sided Fisher's exact test (Zeeburg et al., 2003). The GoMiner analysis reflected the hypothesis that gene expression would not be significantly different between the left and right hippocampus at E18. Thus, similar to the SAM analysis developed for individual genes, the two-sided Fisher's exact test was used in GoMiner to determine whether a particular gene ontology category was significantly enriched based on the individual genes that were significantly lateralized as compared to all of the genes within that category represented on the microarray (Zeeburg et al., 2003).

Gene Set Enrichment Analysis (GSEA) of Microarray Data: Gene sets are defined based on previously published information on biochemical pathways or gene ontology categories and are used to determine whether genes within a given set are more likely observed at a specific location within a list to compute an enrichment score. The enrichment score reflects the degree to which a gene set is observed at the top of the ranked list based on the Kolmogorov-Smirnov statistic (Hollander and Wolfe, 1999; Subramanian et al., 2005).

Quantitative Real Time PCR Analysis (qRT-PCR): For gene expression assays using quantitative real-time RT-PCR, total RNA from the right or left hippocampal formation of individual E18 rats was isolated and purified. Reverse transcription of 0.5 µg of DNased total RNA from the left or right hippocampal formation was performed by priming with oligo(dT) and random hexamers, utilizing SuperScriptIII (Invitrogen,

Carlsbad, CA). A 1:10 dilution of cDNA was used as a template for RT-PCR using Brilliant SYBR Green qRT-PCR Master Mix (Stratagene) on an Mx3000P Real-Time PCR System. ROX reference dye was used in all reactions. Primer sets were optimized for each gene across intron: exon boundaries to derive approximately 100 base-pair amplicons. The final amplification conditions were optimized based on the T_m and concentration of the individual primer sets that were initially assessed using gel electrophoresis. In addition, dissociation curves were performed on all reactions to assure product purity. Original RNA amounts were determined by comparison to standard curves. Experiments were performed in triplicate for each data point (the left or right hippocampi of individual male rats at E18; n = 11).

Western Blot Analysis: A total of 4 μg of lysate protein from each sample (the left or right hippocampi of individual male rats at E18; n = 10) was electrophoresed through 10% polyacrylamide gels and electrophoretically transferred to PVDF membranes (Millipore, USA) in a buffer containing 25 mM glycine, 192 mM Tris base, and 20% methanol. Membranes were blocked for 1 hour at 25°C in a buffer containing 1% nonfat dry milk, 1% BSA, 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween 20 followed by incubation in an appropriate dilution of α1a-tubulin primary antibody (rabbit polyclonal antibody, Santa Cruz Biotechnology, USA) overnight at 4˚C. Subsequent to room temperature incubation in horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit secondary antibody, Santa Cruz Biotechnology) for 1 hour, the immunoreactive band was visualized on film (BioMax, Kodak, USA) by enhanced chemiluminescence (ECL) detection (Amersham) and quantitated using ImageJ software

(http://rsb.info.nih.gov/ij/). The membranes were stained with Ponceau S (Sigma, USA) to ensure equal protein loading.

RESULTS

Differential gene expression was observed during embryonic development of the rat hippocampal formation and all of the differentially expressed genes were more highly expressed in the right hippocampus at E18. As described above, I used a microarray to assay the expression of 1,178 genes (specific to the rat brain and representing greater than 90% of the ontological categories) and SAM paired analysis with a false discovery rate (q-value) of less than 10%, to compare gene expression levels in the left and right hippocampi of the male rat at E18. Fourteen genes were differentially expressed and all were more highly expressed in the right hippocampus–indicating a right dominance in gene expression at E18 (Table 5.1).

The fold change values determined using SAM (Tusher et al., 2001; Van de Wiel et al., 2004) were indicative of the percent increase in gene expression in one hemisphere relative to another (Table 5.1). Thus, fold change values greater than 1.00 reflected greater gene expression in the right hippocampus, whereas, fold change values less than 1.00 specified greater gene expression in the left hippocampus. For example, beta3-tubulin had a fold change value of 1.22, thus, expression was 22% higher in the right hippocampus as compared to the left. The false discovery rate, or q-value, as determined by SAM is used to address the problem of multiple comparisons when examining a large set of genes in a microarray by calculating the probability that a given gene within the set is a false positive – when the gene is not actually differentially expressed even when the fold change values indicate that is the case. For example, beta3-tubulin has a false

discovery rate of less than 0.00; thus, there is a very high probability that this gene is more highly expressed in the right hippocampus at E18. In contrast, alpha1a-tubulin had a false discovery rate of 9.96 indicating that there was a 9.96% chance that the 14% increase in alpha1a-tubulin gene expression in the right hippocampal formation at E18 was a false positive.

Table 5.1. ***Differentially expressed genes were more highly expressed in the right hippocampus at E18.***

Gene Bank Accession No.	Gene Name	Fold Change	False Discovery Rate (q-value)
AF459021	tubulin, beta 3	1.22	0.00
X62085	hypoxanthine phosphoribosyltransferase 1	1.22	7.24
X15013	ribosomal Protein L7a	1.20	0.00
AB015946	tubulin, gamma 1	1.18	7.24
AB011679	tubulin, beta 5	1.16	0.00
M19533	peptidylprolyl isomerase A	1.16	0.00
U03390	G protein, beta polypeptide 2 like 1	1.15	0.00
U53211	amiloride-sensitive cation channel 1	1.14	0.00
V01227	tubulin, alpha 1a	1.14	9.96
X03475	ribosomal protein L35a	1.14	0.00
X02231	GAPDH	1.13	7.24
X73683	H3 histone, Family 3B	1.13	7.24
NM_012512	beta-2 microglobulin	1.09	7.24
S53987	nACh Receptor, alpha 7 subunit	1.08	7.24

Fold change values greater than 1.00 are indicative of higher levels of gene expression in the right relative to left hippocampus (n=20 male rats at E18 from 5 litters) using SAM paired analysis with a false discovery rate (q-value) of less than 10%.

Gene ontology categories related to hippocampal growth and development were enriched in the right hippocampus at E18. Simply compiling a list of significantly enriched genes, like those included in Table 5.1, could lead to a somewhat arbitrary focus on single genes within the list (Hosack et al., 2003; Subramanian et al., 2005). Therefore, DAVID Gene Functional Annotation Analysis was used to examine gene enrichment for

three gene ontology categories: molecular function, cellular components, and biological processes (Dennis et al., 2003; Huang et al., 2009). Forty-two gene ontology terms (GoTerms) within those gene ontology categories were significantly enriched in the right hippocampus at E18 (Appendix A). Of the 42 GoTerms that were enriched in the right hippocampus, 13 were in the molecular function category, 15 in the cellular components category, and 14 in the biological processes category. The identified GoTerms indicated that certain biological processes, such as neuronal structure, growth and differentiation, cellular metabolism, and protein synthesis were upregulated in the right hippocampus at E18 (Appendix A).

Tubulin genes were enriched in the right hippocampus at E18. I used DAVID Gene Functional Classification Analysis to determine whether any of the 14 genes shown to be differentially expressed at E18 (Table 5.1) could be grouped into categories that would indicate that a set of genes is enriched in the right hippocampus (Huang et al., 2007). While SAM allows for the identification of differentially-expressed genes individually, ontological analyses, such as those utilized in DAVID analysis, are used to determine whether any of the differentially-expressed genes are functionally related. To accomplish this, DAVID Gene Functional Classification Analysis p-values, previously called EASE scores, were calculated using a modified Fisher Exact test (Hosack et al., 2003). Of the 1,178 genes present on the array, only a subset belong to a given gene-classification group. The Fisher Exact test accounts for the number of genes within that gene-classification group identified and the probability of randomly sampling that number

within the entire set. The final enrichment score represents the negative log of the geometric means of the p-values for the genes within the set (Hosack et al., 2003).

Following, DAVID Gene Functional Classification Analysis, the 4 tubulin genes within the original set of 14 genes were found to be significantly enriched in the right hippocampus at E18 (Table 5.2). The enrichment of the tubulin genes within the original set of 14 genes indicated that the further examination of the differential expression of tubulin genes was warranted.

Table 5.2. ***Specific genes enriched in the right hippocampus at E18 using DAVID Gene Function Classification Analysis.***

Gene Bank Accession No.	Gene Name
AB015946	tubulin, gamma 1
V01227	tubulin, alpha 1
AB011679	tubulin, beta 5
AF459021	tubulin, beta 3
Enrichment Score: 2.68	

Because DAVID analysis may overlook some enriched GoTerms that can be observed using other types of ontological analysis (such as GoMiner and GSEA), especially with small gene lists (Khatri et al., 2005; Huang et al., 2009). For this reason, I also examined the gene set identified by paired SAM analysis with less than a 10% false discovery rate using GoMiner analysis (Zeeburg et al., 2003; Zeeburg et al., 2005). I found 155 GoTerms were enriched in the right hippocampus at E18 (Appendix B). As with DAVID analysis, the GoMiner analysis also indicated that gene ontology categories related to growth and development were significantly enriched in the right hippocampus at E18.

In comparing the DAVID and GoMiner results, I found that 27 of the GoTerms were the same on both lists. Of those 27 GoTerms, 10 were in the molecular function category, 11 in the cellular components category, and 6 in the biological processes category. Interestingly, the identified GoTerms from both DAVID and GoMiner analyses included 4 related to the cytoskeleton (cytoskeleton, cytoskeletal part, cytoskeleton-dependent intracellular transport, and the structural constituent of cytoskeleton) and another 4 in common were related to microtubules (microtubule, microtubule cytoskeleton, microtubule based movement, and microtubule-based process). Thus, lateralization of genes in these categories may be of particular interest in the development at E18.

Genes shown to be differentially expressed during embryonic development of the hippocampal formation are also differentially expressed during early postnatal development. As well as determining the directional preference of lateralized gene expression at E18, I also compared my present data with our previous findings of lateralized gene expression during early postnatal development in rats (Moskal et al., 2006). Our lab observed lateralized gene expression as early as P6. At this time point, all of the differentially-expressed genes were more highly expressed in the right hippocampal formation. Of the 14 genes more highly expressed in the right hippocampal formation at E18, three were also more highly expressed in the right hippocampal formation at P6 (Table 5.3; Moskal et al., 2006). Thus, some of the genes that are more highly expressed at E18 remained more highly expressed at P6, whereas the remaining 11 were not differentially expressed at this later time point. Our lab has also previously shown a right-to-left shift in lateralized gene expression between P6 and P9 where the

majority of the differentially-expressed genes were more highly expressed in the left hippocampal formation at P9 (Moskal et al., 2006). Interestingly, of the genes shown to be preferentially expressed in the right hippocampus at E18, half (7/14) were more highly expressed in the left hippocampal formation at P9 (Table 5.3; Moskal et al., 2006).

Table 5.3: ***Changes in the differential expression of genes lateralized at E18 at the end of the first postnatal week***

Gene Bank Accession No.	Gene Name	E18	*P6	*P9
U03390	G protein, beta polypeptide 2 like 1	R	R	-
V01227	tubulin, alpha 1a	R	R	L
X02231	GAPDH	R	R	L
L27487	peptidylprolyl isomerase A	R	-	-
NM_012512	beta-2 microglobulin	R	-	-
S53987	nACh Receptor, alpha 7 subunit	R	-	-
U53211	amiloride-sensitive cation channel 1	R	-	-
X03475	ribosomal protein L35a	R	-	-
X73683	H3 histone, Family 3B	R	-	-
AB011679	tubulin, beta 5	R	-	L
AB015946	tubulin, gamma 1	R	-	L
AF459021	tubulin, beta 3	R	-	L
X15013	ribosomal Protein L7a	R	-	L
X62085	hypoxanthine phosphoribosyltransferase 1	R	-	L

SAM analysis (FDR<10% at E18, P6, and P9 during normal development). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=20 male rats at E18 from 5 litters; n=6 from 3 litters at P6 and P9). * Data from Moskal et al. (2006).

Genes corresponding to proteins that comprise signaling pathways related to growth and development were more highly expressed in the right hippocampus at E18 and later shifted to be more highly expressed in the left by P9. DAVID analysis was further utilized to more closely examine signaling pathways comprised of the specific genes that were differentially expressed at E18 (Table 5.1) and during early postnatal development (Moskal et al., 2006). Genes corresponding to proteins that comprise signaling pathways related to cellular structure (Table 5.4), transcription and translation (Table 5.5), and gap

junction signaling (Table 5.6) were more highly expressed in the right hippocampus at E18 and shifted to be more highly expressed in the left hippocampal formation by P9. Of the 5 genes related to cellular structure, 4 were more highly expressed in the right hippocampus at E18 and one of those remained more highly expressed in the right at P6; all 5 of the genes were more highly expressed in the left hippocampal formation at P9 (Table 5.4). Of the 4 genes related to transcription and translation, 3 were more highly expressed in the right hippocampus at E18 and an additional gene was more highly expressed in the right hippocampus at P6. In contrast, at P9 only one of the 4 genes was more highly expressed in the left hippocampal formation and the remainder showed no directional preference (Table 5.5). Of the 5 genes related to gap junction signaling, 3 were more highly expressed in the right hippocampus at E18 and two genes were more highly expressed in the right at P6 whereas at P9, 4 of the genes were more highly expressed in the left hippocampal formation (Table 5.6). Thus, genes within these pathways that were initially differentially expressed at E18 continued to be differentially expressed during postnatal development. Interestingly, most of those genes were tubulin genes.

Table 5.4: ***Genes related to cell structure that were differentially expressed during embryonic and early postnatal development in the rat hippocampal formation.***

Gene Bank Accession No.	Gene Name	E18	*P6	*P9
Structure				
V01227	tubulin, alpha 1a	R	R	L
AB011679	tubulin, beta 5	R	-	L
AB015946	tubulin, gamma 1	R	-	L
AF459021	tubulin, beta 3	R	-	L
V01217	actin, beta	-	-	L

SAM analysis (FDR<10% at E18, P6, and P9 during normal development). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=20 male rats at E18 from 5 litters; n=6 from 3 litters at P6 and P9). * Data from Moskal et al. (2006).

Table 5.5: ***Genes related to transcription and translation that were differentially expressed during embryonic and early postnatal development in the rat hippocampal formation.***

Gene Bank Accession No.	Gene Name	E18	*P6	*P9
Transcription and translation				
X03475	ribosomal protein L35a	R	-	-
X15013	ribosomal Protein L7a	R	-	L
X73683	H3 histone, Family 3B	R	-	-
D25224	40S ribosomal protein SA	-	R	-

SAM analysis (FDR<10% at E18, P6, and P9 during normal development). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=20 male rats at E18 from 5 litters; n=6 from 3 litters at P6 and P9). * Data from Moskal et al. (2006).

Table 5.6: ***Genes related to gap junction signaling that were differentially expressed during embryonic and early postnatal development in the rat hippocampal formation.***

Gene Bank Accession No.	Gene Name	E18	*P6	*P9
Gap Junction Signaling				
V01227	tubulin, alpha 1a	R	R	L
AF459021	tubulin, beta 3	R	-	L
AB011679	tubulin, beta 5	R	-	L
X04139, K03486	protein kinase C, beta 1	-	R	-
L14323	phospholipase C, beta 1	-	-	L

SAM analysis (FDR<10% at E18, P6, and P9 during normal development). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=20 male rats at E18 from 5 litters; n=6 from 3 litters at P6 and P9). * Data from Moskal et al. (2006).

Additionally, at E18, P6, and P9, genes encoding proteins related to cellular metabolism (Table 5.7) and glycolysis (Table 5.8) were differentially expressed. Although the same individual genes were not always differentially expressed in each age group, genes within the same signaling pathway often showed similar patterns of expression during early development. Only hypoxanthine phosphoribsyltransferase 1, which is involved in cellular metabolism, was more highly expressed in the right hippocampus at E18 and shifted to be more highly expressed in the left hippocampal formation by P9 (Table 5.7). However, an additional 3 genes involved in cellular metabolism were more highly expressed in the right hippocampal formation at P6 and an additional 4 genes were more highly expressed in the left hippocampal formation at P9 (Table 5.7). Furthermore, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a component of the glycolysis signaling pathway was more highly expressed in the right hippocampus at E18, remained more highly expressed in the right hippocampal formation at P6, and then shifted to be more highly expressed in the left hippocampal formation at

P9 (Table 5.8). An additional 4 genes in the glycolysis pathway were also more highly expressed in the left hippocampal formation at P9 (Table 5.8), but were not differentially expressed prior to that point in development.

In examining the pattern of lateralized gene expression during hippocampal development, it is important to note that, although only 1 gene in the cellular metabolism pathway or the glycolysis pathway, was differentially expressed at E18 a pattern of expression emerged when the differentially-expressed genes within the pathway were examined at different points across development. Those genes that were differentially expressed were more highly expressed in the right hippocampal formation until P6 and then shifted to be more highly expressed in the left hippocampal formation at P9.

Table 5.7: ***Genes related to cellular metabolism that were differentially expressed during embryonic and early postnatal development in the rat hippocampal formation.***

Gene Bank Accession No.	Gene Name	E18	*P6	*P9
Cellular Metabolism				
X62085	hypoxanthine phosphoribosyltransferase 1	R	-	L
M23601	monoamine oxidase B	-	R	-
X07467	glucose-6-phosphate dehydrogenase	-	R	-
S73424	macrophage migration inhibitory factor	-	R	-
NM_012734	hexokinase 1	-	-	L
U73859	hexokinase 3	-	-	L
M68971	hexokinase 2	-	-	L
J04218	Glucokinase	-	-	L

SAM analysis (FDR<10% at E18, P6, and P9 during normal development). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=20 male rats at E18 from 5 litters; n=6 from 3 litters at P6 and P9). * Data from Moskal et al. (2006).

Table 5.8: ***Genes related to glycolysis that were differentially expressed during embryonic and early postnatal development in the rat hippocampal formation.***

Gene Bank Accession No.	Gene Name	E18	*P6	*P9
Glycolysis				
X02231	GAPDH	R	R	L
J04218	Glucokinase	-	-	L
NM_012734	hexokinase 1	-	-	L
M68971	hexokinase 2	-	-	L
U73859	hexokinase 3	-	-	L

SAM analysis (FDR<10% at E18, P6, and P9 during normal development). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=20 male rats at E18 from 5 litters; n=6 from 3 litters at P6 and P9). * Data from Moskal et al. (2006).

Genes corresponding to proteins that comprise the gap junction signaling pathway were more highly expressed in the right hippocampus of the rat at E18. The gap junction signaling pathway was identified as being significantly enriched in the right hippocampus of the rat at E18 (p = 0.018) using DAVID analysis (Dennis et al., 2003; Huang et al., 2009). GSEA analysis was used to further examine the enrichment of genes within the gap junction signaling pathway, the specific genes shown to be enriched in the gap junction signaling pathway were alpha-tubulin, beta-tubulin, Ras, and PKC (p=0.038; Figure 5.1). Furthermore, as noted above, alpha- and beta-tubulin were more highly expressed in the right hippocampus as determined by SAM analysis with a false discovery rate of less than 10% (Table 5.1) and were significantly enriched in the right hippocampus as determined by DAVID analysis (Table 5.2). For these reasons, the expression of alpha- and beta-tubulin was further studied using quantitative real-time RT-PCR (qRT-PCR) in the rat hippocampus at E18.

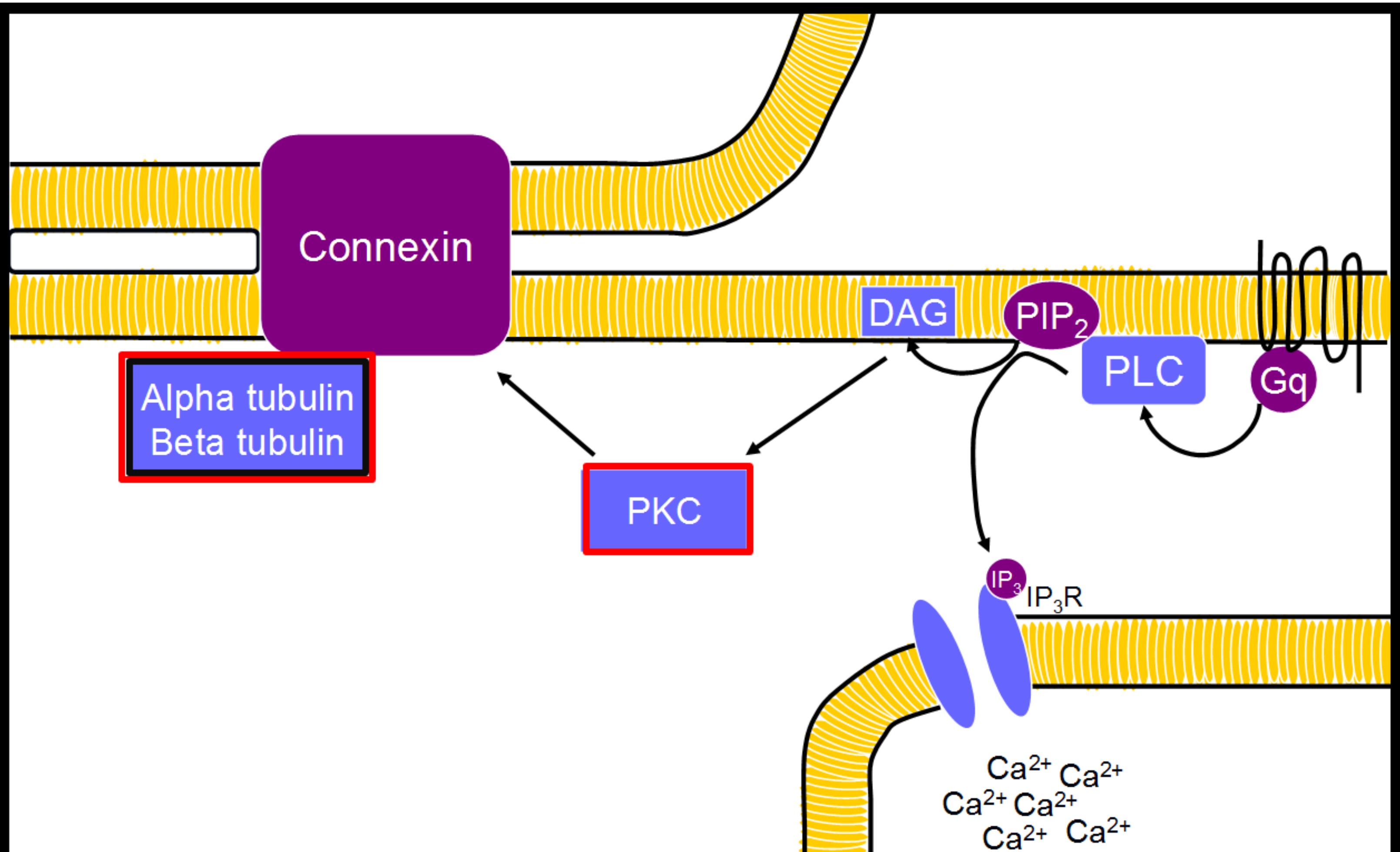

Figure 5.1: ***Gap Junction Signaling Pathway.*** Genes shown to be differentially expressed at E18 using SAM analysis with a false discover rate of less than 10% are boxed in black (see table 5.1) and those enriched in the right hippocampus at E18 following GSEA analysis (p=0.038) are also boxed in red.

It is important to note that not all of the genes in the gap junction pathway were examined in the original microarray analysis. For example, connexin43 is an integral component of the gap junction pathway, but was not present on the original array (Kroes et al., 2006). Raya and Belmonte (2006) have previously argued for specifically examining the role of connexin43 in the establishment of left-right asymmetry and, interestingly, connexin43 has also been shown to be differentially expressed in the human during embryonic development (Sun et al., 2006). For this reason, the lateralized expression of connexin43 mRNA at E18 was also examined using qRT-PCR.

Thus, expression of 3 genes that comprise the gap junction pathway were examined using qRT-PCR: alpha1a-tubulin, beta3-tubulin, and connexin43. Alpha1a-tubulin was significantly more highly expressed in the right hippocampus (2.03 ± 0.26, mean ± SEM) as compared to the left (1.64 ± 0.11; p = 0.04; Figure 5.2). Beta3-tubulin was also more highly expressed in the right hippocampus (0.033 ± 0.004) as compared to the left (0.024 ± 0.004, p=0.03; Figure 5.3). Additionally, connexin43 was more highly expressed in the right hippocampus at E18 (0.011 ± 0.001) as compared to the left (0.009 ± 0.002; p = 0.04; Figure 5.4). Thus, alpha1a-tubulin, beta3-tubulin, and connexin43 were all more highly expressed in the right hippocampus of the rat at E18. Importantly, the identification of lateralized expression of genes selected by function using GoTerm and DAVID analysis led to the discovery that Connexin43 – not probed on the original array, was indeed more highly expressed in the right hippocampal formation at E18. This result validates the microarray data.

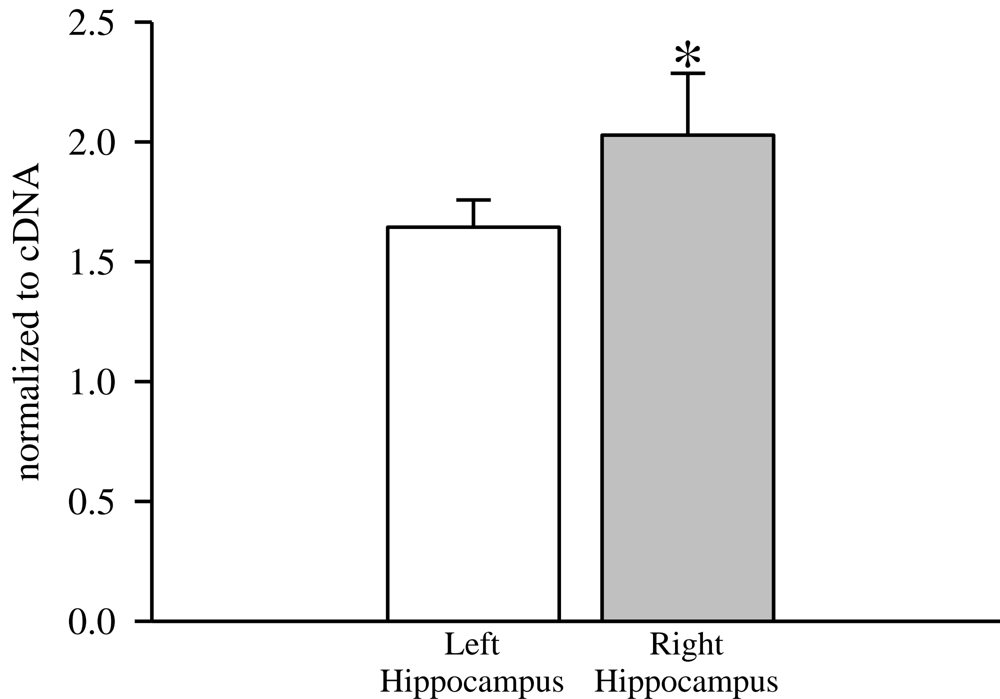

Figure 5.2: *Expression of Alpha1a-tubulin mRNA in the rat hippocampus at E18.* Alpha1a-tubulin mRNA was more highly expressed in the right hippocampus of the male Sprague-Dawley rat at E18 (*p = 0.04). Transcript abundance was normalized to cDNA yield (n = 11 rats from 5 litters).

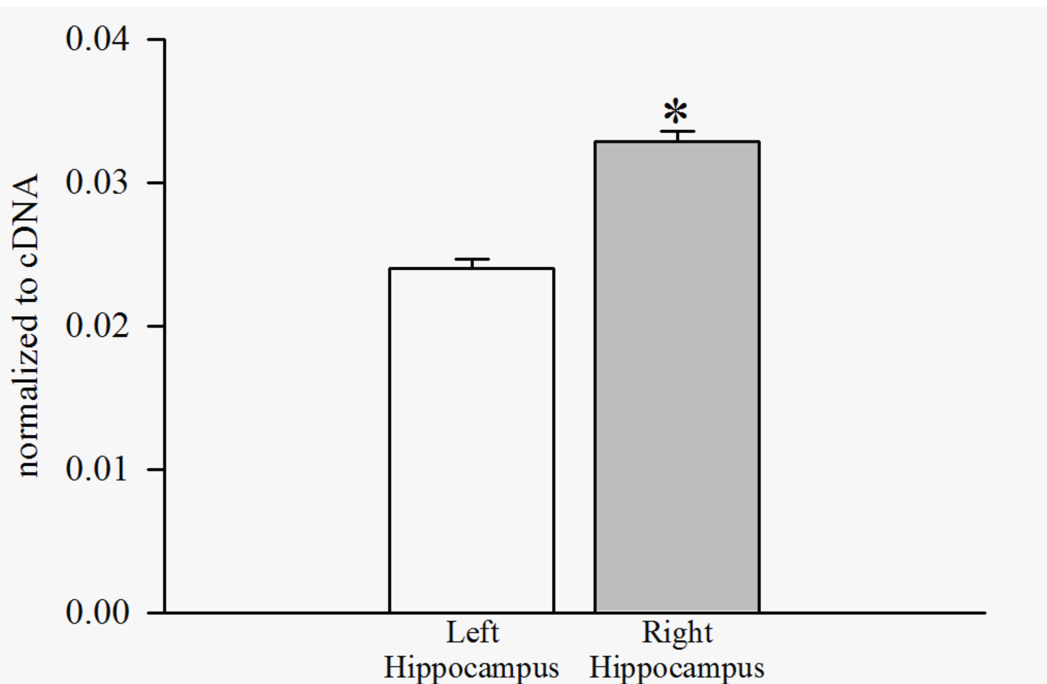

Figure 5.3: *Expression of Beta3-tubulin mRNA in the rat hippocampus at E18.* Beta3-tubulin mRNA was more highly expressed in the right hippocampus of the male Sprague-Dawley rat at E18 (*p = 0.03). Transcript abundance was normalized to cDNA yield (n = 11 rats from 5 litters).

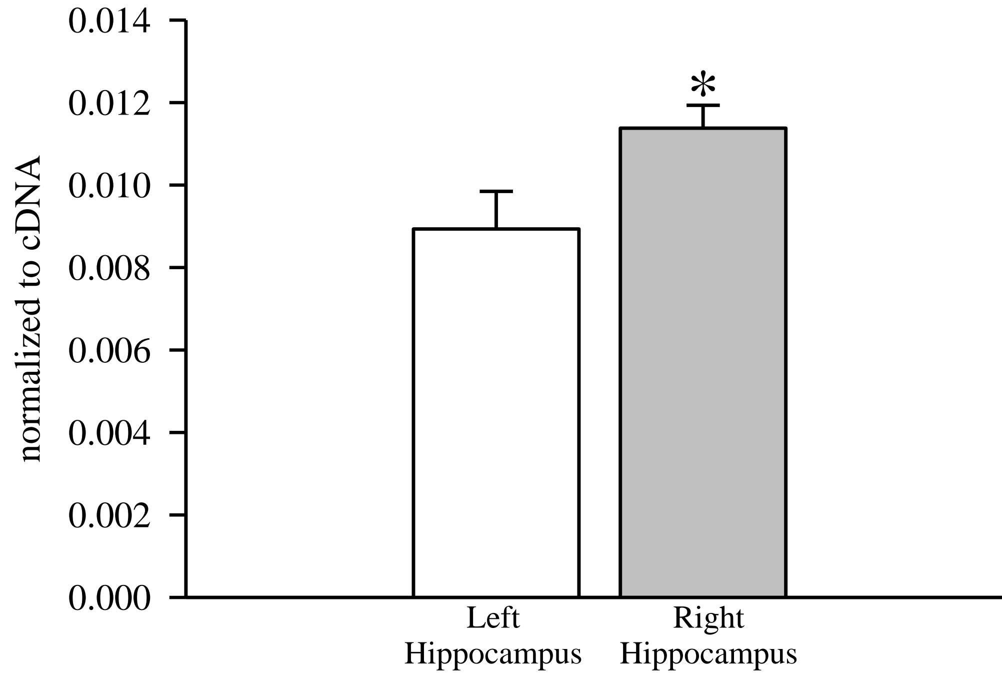

Figure 5.4: *Expression of Connexin43 mRNA in the rat hippocampus at E18.* Connexin mRNA was more highly expressed in the right hippocampus of the male Sprague-Dawley rat at E18 (*p = 0.04). Transcript abundance was normalized to cDNA yield (n = 11 rats from 5 litters).

To determine whether protein levels of alpha1a-tubulin were also lateralized at E18, western blot analysis was utilized. While microarrays and qRT-PCR are useful for examining mRNA expression, biological processes are normally driven by proteins. To ascertain whether the lateralized gene expression patterns described above were correlated with changes in protein levels, alpha1a-tubulin protein levels were examined using western blot analysis. Results showed that alpha1a-tubulin protein levels were higher in the right hippocampus (1.31 ± 0.23, mean ± SEM) as compared to the left (1.00 ± 0.18; *p =0.02) by approximately 31% (Figure 5.5). It is interesting to note that alpha1a-tubulin mRNA expression was 24% greater (Figure 5.2) in the right hippocampus at E18. Thus, as expected, alpha1a-tubulin gene and protein levels were indeed both greater in the right hippocampus at E18.

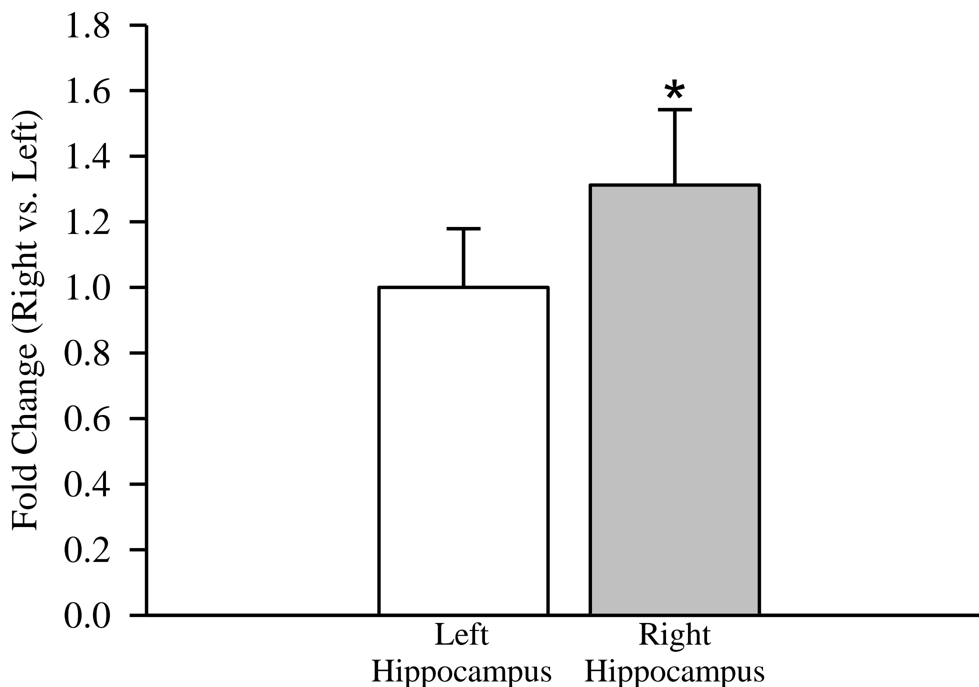

Figure 5.5: ***Alpha1a-tubulin protein levels in the rat hippocampus at E18.*** Alpha1a-tubulin protein levels were greater in the right hippocampus of the male Sprague-Dawley rat at E18 (*p = 0.02, n = 11 rats from 2 litters).

DISCUSSION

The use of gene expression and protein level analysis in the present study allowed for the examination of lateralization in the hippocampus during embryonic development. Results indicated that lateralization of gene expression in the hippocampus is observed at E18. Of the 14 genes that were differentially expressed, all were more highly expressed in the right hippocampus. Those 14 genes comprised signaling pathways important to development, including, cell structure, transcription and translation, cellular metabolism, glycolysis, gap junction signaling. Interestingly, in examining the pattern of lateralized gene expression at E18 and comparing it to our previous findings during early postnatal development (Moskal et al., 2006), I found that preferential gene expression in the right hippocampal formation is observed as early as E18, and is likely maintained until P6 before shifting to be preferentially expressed in the left hippocampal formation by P9.

My results indicated that lateralized gene expression was observed during embryonic development of the rat hippocampal formation. These findings are both novel and important: they are the first to suggest that lateralization of the brain is established during embryonic development in the rat. This finding is supported by previous studies indicating that lateralized gene expression was found in other regions of the human neocortex during gestation (Sun et al., 2005, 2006). Using SAGE analysis, Sun and colleagues (2005) detected lateralized gene expression during embryonic development of the frontal, perisylvian, and occipital cortices at gestational weeks 12 and 14. Thus, lateralized gene expression has been observed during both human and rat development.

In contrast to my present findings, others have argued that lateralization only emerges during postnatal development as a result of environmental influence, rather than being

established prior to birth (Denenberg 1983; 1986; 2005). For example, lateralization of volume (Verstynen et al., 2001) and synaptic activity (Tang et al., 2008) in the hippocampal formation is only observed in adult rats exposed to novelty during the first three postnatal weeks, and, importantly, lateralization was not observed in control rats. While these findings indicate that lateralization of the rat hippocampal formation is only observed following changes in postnatal development, it is important to note that Sun and colleagues (2005, 2006) observed lateralized gene expression in the human embryonic neocortex and the present findings clearly indicated that lateralized gene expression was observed in the hippocampal formation of rat at E18. Thus, these findings contradict the arguments made previously by Denenberg (1983; 1986; 2005) that lateralization is only observed following behavioral manipulation during development.

Additionally, my results indicated that all of the differentially-expressed genes were more highly expressed in the right hippocampus at E18. This clear directional preference in lateralized gene expression has not been previously observed during embryonic development. In humans, Sun and colleagues (2005) found that all of the differentially expressed genes were not more highly expressed in a single hemisphere at gestational weeks 12 and 14. At week 12 of human embryonic development, 51% of the differentially expressed genes were in the frontal region, 57% in the perisylvian region, and 26% in the occipital region were more highly expressed in the right hemisphere. By gestational week 14, only 38% of the differentially expressed genes in the frontal region, 44% in the perisylvian region, and 38% in the occipital region were more highly expressed in the right hemisphere. One reason that Sun and colleagues (2005, 2006) may not have observed a clear directional preference in lateralized gene expression in each

region during human embryonic development is that they examined multiple brain regions in each sample. For example, they dissected the entire left or right frontal region, which would include more than a single brain region, including those anterior to the central sulcus. The inclusion of multiple brain regions in each sample is potentially significant as region-specific gene expression during development has been observed (Wirtz and Schuelke, 2011). Thus, from Sun and colleague's data, one cannot determine whether a clear directional preference in lateralized gene expression is observed within a single brain region at either week 12 or 14 of human embryonic development. Further studies would need to be conducted in order to conclusively determine whether similar pattern of lateralized gene expression are observed in humans and rodents.

Of the individual genes that were shown to be differentially expressed at E18, many are important to the development of the hippocampal formation. For example, the gene for the alpha7 subunit of the nicotinic acetylcholine receptor was more highly expressed in the right hippocampus at E18. During development, acetylcholine has been shown to regulate grown and differentiation of cells within the developing nervous system (Lauder and Schamba, 1999). The hippocampus receives afferent input from cholinergic neurons in the basal forebrain. Prenatal choline supplementation lowered LTP induction threshold (Pyapali et al., 1998) and improved performance in a spatial memory tasks (Meck and Williams, 1999) in rats. The differential expression of the alpha 7 subunit of the acetylcholine receptor during embryonic development could have potentially significant consequences to the later function of the hippocampal formation.

While focusing on the lateralized expression of a single gene, or a subset of genes from a list, may be sufficient to identify molecular mechanisms that provide insight into

biological function, problems can arise when attempting to objectively evaluate a gene list (Subramanian et al., 2005; Subramanian et al., 2007). An important point made by Subramanian and others (2005) is that small fold changes in genes encoding members of a pathway may be more biologically important than a larger fold increase of a single gene. For this reason I utilized ontological analyses to identify pathways composed of genes within the original set of 14 genes (Dennis et al., 2003; Huang et al., 2009; Zeeburg et al., 2005; Subramanian et al., 2005; Subramanian et al., 2007). DAVID analysis allowed for the examination of numerous pathways and the enrichment of specific classes of genes to identify particular pathways of interest. However, DAVID analysis has been criticized for overlooking some enriched terms that may be observed using other types of ontological analysis, especially with small gene lists (Khatri et al., 2005; Huang et al., 2009). For this reason, I also utilized GoMiner and GSEA to identify gene ontology categories and pathways that were significantly enriched in the right E18 male rat hippocampus. While both GoMiner and GSEA are used to extend observations from a simple list of differentially expressed genes to identify pathways and processes, GSEA analysis, unlike GoMiner, allows for the examination not just of the ranked list but of all the genes on the microarray (Subramanian et al., 2005). Many of the pathways identified by DAVID, GoMiner, and GSEA analyses are important to development.

Several tubulin isoforms were found to be more highly expressed in the right hippocampus at E18, including alpha1a-tubulin, beta3-tubulin, beta5-tubulin, and gamma-tubulin and these are known to be important to the structural development of the brain. Microtubules are polymers of alpha and beta tubulin that act as a major component of the cytoskeleton involved in the growth of axons (Zakharenko and Popov, 1998) and

dendrites (Scott and Luo, 2001). More specifically, B3-tubulin, a tubulin gene more highly expressed in neurons during early neuronal differentiation (Lee et al.. 1990; Dennis et al., 2002; Ambrogini et al., 2004), was one of the tubulin genes shown to be more highly expressed in the right hippocampus at E18. Additionally, microtubules have also been shown to be necessary for proper neuronal migration during cortical development (Keays et al., 2007). The differential expression of the tubulin genes could indicate that these processes are upregulated in the right hippocampus during embryonic development.

In addition to tubulin genes, histone (H3) and ribosomal (L7a, L35a) genes known to correspond to proteins involved in transcription and translation are more highly expressed in the right hippocampus at E18. The amount of protein present in cells is determined primarily by the rate of transcription (reviewed in Purves et al., 2004). As genes corresponding to proteins involved in both transcription and translation were differentially expressed at E18, these findings could indicate that rates of protein formation are higher in the right hippocampus during embryonic development.

Tubulin genes also bind directly to Connexin-43 (Giepmans et al., 2001a, 2001b), and microtubules may be necessary for the regulation of connexin43 gap junction signaling (Giepmans et al., 2001a, 2001b). The findings of the present study specifically indicate that the Connexin 43 gene that corresponds to a protein component of a gap junction, which are arrays of intercellular channels formed by connexin proteins that link adjacent cells to allow diffusion of ions and signaling molecules (Giepmans et al., 2001a) were enriched in the right hippocampus at E18. The alpha1a-tubulin, beta3-tubulin, and Connexin43 genes, all corresponding to proteins that are components of the gap junction

signaling pathway, were shown to be more highly expressed in the right hippocampus at E18.

Importantly, communication through gap junctions is a major signaling mechanism during embryonic development (Rozental et al., 1998) as neurochemical signaling pathways develop later in the hippocampal formation (Ben-Ari et al., 2002). Gap junction signaling has been shown to be involved in neurogenesis and is also necessary for proper cellular growth and maturation of synaptic connections (Sutor, 2002; Bruzzone and Dermietzel et al., 2006; Fushiki and colleagues 2006). Fushiki and colleagues (2006) found that in connexin43 null mutant mice neurogenesis and neuronal migration were delayed throughout embryonic development. Connexin43 is the most widely expressed connexin in embryonic development; thus, preferential Connexin43-mediated gap junction signaling in the right hippocampus at E18 may result in increased cell division and migration in the right hippocampus.

In addition to examining lateralized gene expression, I also examined lateralized protein levels of alpha1a-tubulin at E18. Both mRNA and protein levels were greater in the right hippocampus at E18. Using proteomic analysis, Samara and colleagues (2011) examined differential protein levels in young adult male rats. They found that an abundance of cytoskeletal proteins and proteins involved in cellular metabolism was lateralized: 28 proteins were at higher levels in the right hippocampus and 13 proteins were at higher levels in the left hippocampus. More specifically, they found that alpha and beta3-tubulin levels were greater in the right hippocampus. These data seem in contrast to our labs' previous findings where increased expression of alpha1-, beta3-, and beta5-tubulin in the left hippocampal formation at P9 was observed (Moskal et al., 2006).

High-throughput protein level screening methods, such as those used by Samara and colleagues (2011) are imprecise (Hack, 2004), and for this reason, many still rely on mRNA measurements as an indication of lateralized protein levels (reviewed in Fu et al., 2007). These conflicting data suggest that further examination of lateralized tubulin gene expression and protein levels in the adult is warranted.

Additionally, in comparing my findings to our lab's previous findings (Moskal et al., 2006) I found that half of the genes differentially expressed at E18 later shift to clearly being more highly expressed in the left hippocampal formation at P9. As I mentioned above, many of the genes shown to be differentially expressed at E18 are involved in development. An important implication of the present findings is that the development of the left hippocampal formation may be delayed.

The argument that the development of the left hippocampal formation is delayed is consistent with previous findings of increased growth, development, and activity in the right hippocampus during early development in humans (Teylor et al., 1969; Geshwind and Gallaburda, 1985; Chiron et al., 1995; Thompson et al. 2008). Teylor (1969) examined individuals that developed epilepsy as children and found that those individuals with left temporal lobe lesions developed epilepsy at earlier ages and were most likely to develop intractable epilepsy. Teylor (1969) argued that a seizure-producing insult would be more likely to affect the less functionally active hemisphere; thus, he argued that the functional development of the left temporal lobe was delayed. These findings were later supported by studies of cerebral blood flow using positron emission commuted tomography (PET) during early development in children (Chiron et al., 1995). Chiron and colleagues (1995) found that blood flow was greater in the right hemisphere prior to the

age of 3, and then shifted to the left hemisphere during the 4th year of life and remained higher in the left until at least the 16th year in humans. Specifically within the hippocampal formation, Thompson and colleagues (2008) later observed greater right hippocampal volume in full-term and pre-term human infants. Previous studies have suggested that increased cortical volume was directly correlated with increased neurogenesis (van der Beek et al., 2004). For this reason, Thompson and colleagues (2008) argued that more neurons may be born in the right hippocampus of humans prior to birth. Although these previous findings all pertain to postnatal development in the human, they clearly indicate that lateralization is established in early development and that the development of the left hippocampal formation may be delayed.

Developmentally regulated differential gene expression first observed during embryonic development might provide the foundation for morphological, physiological, and functional lateralization observed during later development and in the adult hippocampus. Left-right differences in gene expression (Moskal et al., 2006), anatomy (Lister et al., 2006), neurochemistry (Kristofikova et al., 2004), neurophysiology (Kawakami et al., 2003), and behavior (Bernasconi-Guastalla et al., 1994; Poe et al., 2000; Klur et al., 2009) have clearly been observed during normal development and in adult rats. Our lab showed that genes involved in synaptic function, morphology, and vesicle trafficking were preferentially expressed in the right hippocampal formation at P6, the left hippocampal formation at P9, and then continued to be more highly expressed in the left hippocampal formation of young adult male rats at P60 (Moskal et al., 2006). Lister and colleagues (2006) found that the CA3/CA2 and CA1 hippocampal regions had a greater number of neurons in the left hemisphere at postnatal day 90.

Kristofikova and colleagues (2004) noted greater high affinity choline uptake in the left, relative to the right, hippocampus at postnatal day 14 in male rats that was sustained until 3 months of age. The N-methyl-D-aspartate (NMDA) receptor ε2 subunit was shown to be lateralized in hippocampal region CA1 pyramidal neurons in mice. The ε2 subunit proteins were greater in the left, as compared to the right, apical dendrites: the opposite was the case for the basal dendrites (Kawakami et al., 2003). Wu et al. (2005) later showed that lateralization of the ε2 subunit was not observed in interneurons. Behavioral studies also indicate that lateralization of the hippocampal formation may be functionally significant in the rodent. Bernasconi-Guastalla and colleagues (1994) showed, using the Morris water-maze, that reversal learning was much faster in mice with larger left intrapyramidal and infrapyramidal mossy fiber axon projections from the dentate gyrus to pyramidal neurons in region CA3 of the hippocampus. In the rat, Poe et al. (2000) showed that inactivation of the left, but not the right, hippocampal formation produced learning and memory deficits of the radial eight-arm maze in aged, but not young, rats. Klur and colleagues (2009) later showed that the left hippocampal formation was necessary for learning during acquisition trials in the Morris water-maze task, whereas the right hippocampal formation was necessary for remembering the location of the platform during the probe trial in adult rats. Thus, lateralization of the hippocampal formation observed in the adult is not dependent upon postnatal changes such as novelty exposure. More likely, environmental influences impact lateralization that is previously established by gene expression, resulting in differential activation of signaling pathways and receptors that would cause long lasting changes in neuronal activity and behavior (Tang et al., 2008).

Furthermore, lateralization of the developing hippocampal formation may be necessary for proper hippocampal function: lateralization of the hippocampal formation has also been implicated in the development of epilepsy (Teyler, 1969), autism (Allman et al., 2005), and schizophrenia (Crow and Harrington, 1994; Harrison., 1999; Zaidel et al., 1999; Spaniel et al., 2003; Hanlon et al., 2005; Hanlon and Sutherland, 2005) and even learning disabilities (Geshwind and Gallaburda, 1985). Thus, an understanding of normal left-right patterning that emerges during hippocampal development is necessary to fully understand changes that occur in learning disabilities, epilepsy, or neuropsychiatric disorders. For example, in schizophrenic individuals, the reduction in hippocampal volume and surrounding areas of the temporal lobe has been shown to be greater in the left hemisphere indicating that changes in lateralization of the hippocampal formation may be important in the development and progression of schizophrenia (Crow and Harrington, 1994; Harrison, 1999). Zaidel et al (1999) closely compared the neuronal morphology of schizophrenic and non-schizophrenic individuals and observed subregion specific changes throughout the hippocampal formation: left CA1 and CA2 pyramidal neurons were smaller, while left CA3 pyramidal neurons were larger, in schizophrenics. A greater variability in CA3 pyramidal neuron orientation was also observed in the right hemisphere of schizophrenics (Zaidel et al., 1999). These findings demonstrate differences in morphology between hemispheres and the potential for differences in neuronal connections within subregions of the hippocampal formation that are differentially affected by schizophrenia. Using magnetoencephalography, Hanlon et al. (2005) showed that schizophrenic individuals exhibit bilateral or greater activity in the left hippocampus in the transverse patterning task, which is hippocampal dependent;

whereas, controls performed better on the task and showed greater activity in the right hippocampal formation. This indicates that lateralized activity is necessary for some forms of learning and memory and that this activity is altered in schizophrenia. Lateralization of the hippocampal formation is not unique to these conditions, but changes in lateralized activity of the hippocampal formation may account for some of the behavioral differences observed. Therefore, it is important to characterize the development of hippocampal lateralization in order to determine when changes to hippocampal lateralization can result in learning and memory deficits or symptoms associated with disease.

Taken together, the present findings indicate that lateralized gene expression in the hippocampal formation is established during embryonic development as early as E18 in the rat, even though the primary period of neurogenesis continues until the end of the second postnatal week and the maturation of the hippocampal formation continues until rats reach adulthood (Altman and Das, 1975; Kaplan and Hinds, 1977; reviewed in Amaral and Lavenoux, 2007; Rahimi and Claiborne, 2007). Additionally, an important implication of my findings is that the development of the left hippocampal formation may be delayed.

CHAPTER 6. EFFECT OF A REDUCTION IN N-METHYL D-ASPARTATE GLUTAMATE RECEPTOR MEDIATED SYNAPTIC ACTIVITY ON LATERALIZED GENE EXPRESSION IN THE RAT HIPPOCAMPAL FORMATION DURING EARLY POSTANTAL DEVELOPMENT

INTRODUCTION

Neurotransmitters are important in the development of the nervous system, including the hippocampal formation (reviewed in Harlenius and Lagercrantz, 2004). Importantly, changes in neurotransmitter levels and receptor types occur during specific stages of development, often termed critical periods, when neurotransmission is necessary for correct development (reviewed in Harlenius and Lagercrantz, 2004). During these critical periods, neurotransmitters are necessary for the development and maturation of synapses that lead to the formation of appropriate neural networks. One such example is the excitatory amino acid glutamate. The number of glutamatergic terminals increases during the perinatal period and remain at high levels until at least the end of the first postnatal week in the rat and this is correlated with an increase in synaptogenesis (reviewed in Harlenius and Lagercrantz, 2004). Additionally, glutamate levels decrease at time points that correspond to the end of critical periods (Constantine-Paton, 1994).

N-methyl-D-aspartate glutamate receptor (NMDAR) density has been shown to increase in the hippocampal formation over the first postnatal week (Baudry et al., 1981; Tremblay et al., 1988; McDonald and Johnston, 1990; Bellone and Nicoll, 2007). In the rat hippocampal formation, NMDAR binding was first observed at P4 and NMDAR density increased over the first postnatal week, reached maximum levels (160% of adult

values) at P9 and then decreased to adult levels by P23 (Baudry et al., 1981). Interestingly, the increase in NMDAR density over the first postnatal week coincides with the time point when NMDAR-dependent long-term potentiation (LTP) is first observed in the hippocampal formation (Harris and Teyler, 1983; Durand et al., 1994; O'Boyle et al., 2004). NMDAR-dependent LTP is first seen in CA1 of the hippocampus at P5 (Harris and Teyler, 1983; Durand et al., 1994) and in the dentate gyrus at P7 when the first adult-like dentate granule neurons are observed (O'Boyle et al., 2004). LTP is a model of learning and memory where memories are represented by changes in synapses within a network of neurons (Bliss and Lomo, 1973), similar to Hebb's postulate where when one neuron persistently takes part in the firing of a second neuron is results in changes in the growth, metabolism, or activity that selectively strengthens the connections between those cells (Hebb, 1949). In that instance the NMDAR acts as a coincidence detector for membrane depolarization and glutamatergic synaptic transmission. This is in contrast to activity at other glutamate receptors, including the AMPA receptor that mediates fast synaptic transmission and only requires binding of glutamate to open. It is important to note that the AMPAR is also required for LTP induction by allowing for depolarization of the cell that removes the magnesium block from the NMDAR.

Importantly, the NMDAR appears to modulate neuronal development in the hippocampal formation during the first postnatal week by affecting the maturation of granule neurons (Sanchez et al., 2001; Jones et al., 2003) and this has been proposed to be important in the formation of normal neural networks through appropriate synapse formation, including those in the hippocampal formation (Constantine-Paton, 1994;

Battistin and Cherubini, 1994; Ben-Ari et al., 1997; Frotscher et al., 2000; Luthi et al., 2001; Bellone and Nicoll, 2007). The NMDAR is thought to play a role in developing neural systems through the stabilization of excitatory synapses following receptor activation and previous investigators have used systemic injections of NMDAR antagonists to simulate the effects of a general reduction in sensory input during development (Sanes et al., 2006; Constantine-Paton, 1994). Previously, our lab showed that the intraperitoneal injection of the NMDAR antagonist 3-((+/-)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP; Harris et al., 1986) blocked long-term potentiation (LTP) induction in dentate granule cells following stimulation of the medial perforant path axons at the end of the first postnatal week *in vivo* (O'Boyle et al., 2004). Furthermore, our lab showed that injections of CPP between P6 and P9 delayed the morphological development of dentate granule neurons in the rat hippocampal formation (Sanchez et al., 2001). A greater percentage of immature neurons (18%) were observed at P9 following CPP injections between P6 and P9 as compared to the percentage (4%) seen in saline-injected control rats, and a smaller percentage of mature granule neurons (11%) were observed at P9 following CPP injections between P6 and P9 as compared to the percentage (31%) seen in saline-injected control rats (Sanchez et al., 2001). Thus, it appears that NMDAR-mediated synaptic activity is necessary for the maturation of dentate granule neurons.

Available data suggest that hippocampal lateralization in the adult rat is influenced by NMDAR-mediated synaptic activity during development (Kristofikova et al., 2004) and that NMDARs are differentially expressed in CA1 of the adult mouse hippocampus (Kawakami et al., 2003; Shinohara et al., 2008). Kirstofikova and colleagues (2004)

found that bilateral, intracerebroventricular injections of quinolinic acid (QUIN), an NMDAR agonist, at P12 reversed lateralization of high affinity choline uptake in male rats. HACU was greater in right hippocampus in adult rats male rats injected with QUIN at P12, rather than being greater in the left hippocampus. Kawakami and colleagues (2003) showed that the ε2 subunit of the NMDAR was more highly expressed in left apical dendrites and right basal dendrites of CA1 pyramidal cells in mice. Additionally, the ε2 subunit of the NMDAR was predominantly located in thin, rather than mushroom, spines (Shinohara et al., 2008). However, the effects of NMDAR-mediated synaptic activity on hippocampal lateralization during early development have yet to be determined.

Importantly, our lab observed lateralized gene expression in the hippocampal formation in the first postnatal week (Moskal et al., 2006). At P6, all of the differentially expressed genes were more highly expressed in the right hippocampal formation, whereas by P9 the majority of the differentially expressed genes were more highly expressed in the left hippocampal formation and continued to be more highly expressed in the left hippocampal formation at P60. Thus, during normal development, a right-to-left shift in lateralized gene expression was observed at the end of the first postnatal week. The right-to-left shift in lateralized gene expression between P6 and P9 coincides with changes in NMDAR-mediated synaptic activity during development of the hippocampal formation. For this reason, I chose to examine the role of NMDAR-mediated synaptic activity on lateralized gene expression in hippocampal development.

The objective of this study was to determine gene expression patterns in the rat hippocampal formation at P9 following a reduction in NMDAR-mediated synaptic

activity between P6 and P9. Here I tested the hypothesis that a reduction in NMDAR-mediated synaptic activity between P6 and P9 with CPP would delay the right-to-left shift in lateralized gene expression normally observed between P6 and P9. In the present study, intraperitoneal injections of CPP were utilized to reduce NMDAR-mediated synaptic activity between P6 and P9.

The results reported in the present study demonstrate that a reduction in NMDAR-mediated synaptic activity between P6 and P9 resulted in a completely novel pattern of lateralized gene expression at P9 that was distinct from the pattern observed during normal development. These results suggest that a reduction in NMDAR-mediated synaptic activity delays the right to left shift in lateralized gene expression normally observed in the hippocampal formation between P6 and P9. Furthermore, these findings indicate that genes corresponding to proteins involved in calcium signaling and signaling pathways regulated by calcium were no longer more highly expressed in the left hippocampal formation at P9 in rats treated with CPP.

METHODS

Animals: Timed-pregnant Sprague-Dawley rats (Charles River Labs, Wilmington, MA) were housed in the University of Texas at San Antonio (UTSA) and the University of New Mexico (UNM) Animal Facilities and provided with food and water *ad libitum* and kept on a 12 hour light dark cycle (n = 7). All procedures were approved by the UTSA and UNM IACUC committee and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The day of birth was considered P0 and all litters were culled to 10 rats on P3.

CPP Injections: In order to examine lateralized gene expression at P9 following a reduction in NMDAR activity between P6 and P9, rats were either injected (intraperitoneal, i.p) with the highly selective NMDA glutamate receptor antagonist CPP at a dosage of 2 mg/kg, or with a comparable volume of saline. Injections were given twice daily, starting on P6 and ending on P9 (Harris et al., 1986; O'Boyle et al., 2004). Two male rats from each litter were sacrificed at P6 to determine whether genes examined were preferentially expressed in the right hemisphere as expected based on our previous findings (Moskal et al., 2006) and to determine whether specific genes examined using qRT-PCR were lateralized at P6. Our lab previously noted slight ataxia following administration of CPP at a dosage of 2 mg/kg (i.p) on postnatal day 6, thereby resulting in a reduced ability to nurse in litters with more than 4 rats. However, it is important to note that culling the litters to 4 young rats allowed CPP-treated animals to nurse as well as in saline-injected littermates and no significant differences were observed in weight between CPP and saline treated littermates (Sanchez et al., 2001; O'Boyle et al., 2004; Rahimi et al., 2006; data not shown). For this reason, litters were culled to 4 male rats on P6 (Jackson, 1912). Any litter with less than 5 male rats was not used. The injections began at 5:00 pm on P6 and the rats were injected (i.p) with either CPP (n = 2 from each litter) or saline (n = 2 from each litter) every 12 hours until 5:00 am on P9.

Hippocampal Dissections: Rats at P6 and P9 were anesthetized with 4% isoflurane (Halocarbon Products, River Edge, NJ) and oxygen at 2 liters per minute using an Ohio style vaporizer (Model 100H, Surgivet/Anesco, Waukesha, WI) until a vigorous tail pinch no longer elicited a response. After the rats were decapitated under deep general

anesthesia, the entire head was placed in RNA*later* (Ambion Inc., Austin, TX) at 4˚C to immediately reduce RNAse activity (Mutter et al., 2004; Vincent and Deutscher, 2009). The meninges and choroid plexus were removed with fine forceps, and the hemispheres were separated. RNA*later* was added again once the brain was completely removed, and then the hemispheres were separated. Each hippocampus was removed from the adjoining cortex by a cut parallel to the hippocampal fissure and transverse cuts at the rostral and caudal ends (Jones et al., 2003). Once the left and right hippocampi were dissected, they were placed in separate 1 ml aliquots of RNA*later*. In order to ensure that dissection order did not influence RNA yield and possible lateralized gene expression patterns, I alternated which hemisphere was dissected first.

RNA Isolation: RNA from individual rats at P6 (n = 8) or P9 (n = 8 male saline-injected rats; n = 8 male CPP-injected rats) was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA) adapted from the single-step RNA isolation method developed by Chomczynski and Sacchi (1987) that utilizes guanidinium-thiocyanate. This method allows for the purification of RNA from lipid-rich tissues, such as those found in the brain, based on the properties of a silica-based membrane that contains a high-salt buffer allowing up to 100 µg of total RNA to bind to the membrane.

First, the left and right hippocampal tissue samples were homogenized for 30 seconds in 1 ml QIAzol Lysis Reagent (Qiagen, Valencia, CA), an a guanidine isothiocyanate buffer that inhibits RNase activity and dissociates nucleoprotein complexes (Damodaran and Kinsella 1983; Chominczynski and Sacchi, 1987). The samples were transferred to a 1.5 ml microcentrifuge tube and 200 µl of chloroform was added and vortexed for 15 seconds. The samples were then centrifuged at 12,000 g for 15 min at 4˚C to allow for

phase separation following centrifugation. The acidic aqueous phase contained mostly RNA rather than DNA or proteins (Chominczynski and Sacchi, 1987). An equal volume of 70% ethanol was added to the aqueous phase to precipitate the RNA (Shapiro 1981; Chominczynski and Sacchi, 1987) and this was immediately added to the silica membrane column and centrifuged to ensure that the silica membrane within the column contained the total RNA from the individual hippocampal tissue samples. The column was washed with RWI buffer (Qiagen, Valencia, CA) to remove any QIAzol Lysis Reagent contaminants in the RNA sample. DNase I was then added to the column for 15 minutes to ensure that no DNA remained in the isolated sample. The column was washed with RPE buffer (Qiagen, Valencia, CA) and allowed to dry in order to remove any traces of ethanol. Total RNA was eluted in 60 µl of RNAse-free water and then quantified using a NanoDrop spectrophotometer (Thermoscientific, Wilmington, DE) by determining the absorbance at 260 nm and stored at -80˚C until future use. It is important to note that dissection order and the hemisphere dissected had no effect on RNA yield or RNA quality as determined by the 260/280 and 260/230 ratios (data not shown).

Microarray Analysis: We assayed the expression of 1,178 genes specific to the rat brain and representing more than 90% of the major gene ontological categories (Kroes et al., 2006) in the left and right hippocampi of rats at P9 (after having been injected with either saline or CPP between P6 and P9). The rat CNS microarray was designed with 45 base pair sequence oligonucleotides complimentary for the 1,178 messenger RNAs (mRNAs) based on the following criteria: 1) minimal secondary structure; 2) minimal homology to other genes in available databases, such as NCBI/EMBL/TIGR; 3) no repeat regions; and 4) defined Tm using ArrayDesigner software (Lockhart et al., 1996; Kroes et al., 2006).

Oligonucleotides were then synthesized using a PolyPlex 96 well oligonucleotide synthesizer (GeneMachines, San Carlos, CA) using phosphoramidite chemistry and printed using an OmniGrid microarrayer (GeneMachines, San Carlos, CA) on an aldehyde glass microscope slide 250μm apart. The accuracy, reproducibility, and specificity of the microarray were evaluated previously (Kroes et al., 2006) using mRNA spiking experiments (Baum et al., 2003).

Total RNA isolated from individual P9 left and right hippocampi was amplified and labeled using a procedure developed by Van Gelder and colleagues (1990). Specifically, reverse transcription of RNA with an oligo(dT) primer with a T7 promoter was followed by *in vitro* transcription in the presence of amino-allyl dUTP. Three microarray slides were used for each individual rat at P9 following either saline (n = 3) or CPP (n=3) injections (Rahimi et al., 2006; Gross et al., 2007). We utilized universal rat reference RNA (Stratagene) in our analyses and treated identical aliquots concurrently with the tissue samples. Experimental (10μg of either left or right hippocampal aRNA labeled with Cy5) and reference (10 μg labeled with Cy3) amplified RNA were combined and hybridized to the microarray slide for 16 hours at 46˚C. Following washes, Cy3 and Cy5 fluorescence hybridization to each spot on the microarray was quantified using a high-resolution confocal laser scanner (Kroes et al., 2006).

After scanning with a confocal laser scanner, significant analysis of microarrays (SAM) was used to determine the number of differentially expressed genes with a false discovery rate of less than 10% (Tusher et al., 2001; van de Wiel, 2004; available at http://www-stat.stanford.edu/~tibs/SAM/). Importantly, if standard t-tests with a p-value cutoff of 0.01 were utilized on the 1,178 genes assayed, 11 genes would be identified by

chance (Tusher et al., 2001; Kroes et al., 2006). To solve this problem SAM was used to determine a false discovery rate score based on a gene-specific t-test of 500 random permutations (Tusher et al., 2001). Fold change values were determined by comparing the observed versus expected relative difference scores, or the ratio of the change in gene expression to the standard deviation.

Database for Annotation Visualization and Integrated Discovery (DAVID) Analysis of Microarray: I utilized DAVID gene functional classification and gene functional annotation tables to examine interrelated genes within the gene list obtained using SAM analysis. Functional classification tables with medium classification stringency were generated to examine genes within the gene set based on functional similarity (Huang et al., 2009). Additionally, I used Functional Annotation Analysis to examine molecular function, cellular components, and biological process gene ontology categories and molecular pathways.

Gene Set Enrichment Analysis (GSEA) of Microarray: Gene sets were defined based on previously published information on biochemical pathways or gene ontology categories and are used to determine whether genes within a given set are more likely observed at a specific location within a list to compute an enrichment score. The enrichment score reflects the degree to which a gene set is observed at the top of the ranked list based on the Kolmogorov-Smirnov statistic (Hollander and Wolfe, 1999; Subramanian et al., 2005).

Quantitative Real Time PCR Analysis (qRT-PCR): For gene expression assays using quantitative RT-PCR, total RNA from the right or left hippocampal formation of individual rats at P6 (n=8) or P9 (n = 8 male saline-injected rats; n = 8 male CPP-

injected rats) was isolated and purified. Reverse transcription of 1 μg of DNased total RNA from the left or right hippocampal formation was performed by priming with oligo(dT) and random hexamers, utilizing SuperScriptIII (Invitrogen, Carlsbad, CA). A 1:10 dilution of cDNA was used as a template for RT-PCR using Brilliant SYBR Green qRT-PCR Master Mix (Stratagene) on an Mx3000P Real-Time PCR System. ROX reference dye was used in all reactions. Primer sets were optimized for each gene across intron:exon boundaries to derive approximately 100 base-pair amplicons. The final amplification conditions were optimized based on the T_m and concentration of the individual primer sets that will be initially assessed using gel electrophoresis. In addition, dissociation curves were performed on all reactions to assure product purity. Original RNA amounts were determined by comparison to standard curves. Experiments were performed in triplicate for each data point.

RESULTS

Saline and CPP injections between P6 and P9 resulted in changes in lateralized gene expression in the developing rat hippocampal formation.

The goal of the present study was to determine the effect of a reduction in NMDAR-mediated activity between P6 and P9 on lateralized gene expression at P9. As described in the Methods section, NMDAR antagonist CPP (2 mg/kg) injections and saline control injections (i.p) between P6 and P9 were utilized to examine changes in gene expression at P9. Following the saline injections between P6 and P9, 76 genes were differentially expressed in the hippocampal formation at P9. Of the 76 genes, 47 genes were more highly expressed in the left and 29 were more highly expressed in the right hippocampal formation (Table 6.1).

Table 6.1: ***Lateralized gene expression in the rat hippocampal formation at postnatal day 9 following saline injections between P6 and P9***			
Gene Bank Accession No.	**Gene Name**	**Fold Change**	**q-value (%)**
Genes more highly expressed in the left hippocampal formation at P9 following saline injections between P6 and P9			
V01227	tubulin, alpha 1a	0.56	0.00
AB015946	tubulin, gamma 1	0.65	0.00
NM_019218	neurogenic differentiation 1	0.69	0.00
NM_017195	GAP 43	0.71	0.00
U01227	serotonin receptor 3A	0.74	0.00
NM_017066	Pleiotrophin	0.74	0.00
M68971	hexokinase 2	0.74	0.00
D28560	ectonucleotide pyrophosphatase/phosphodiesterase 2	0.75	0.00
U38653	IP3 Receptor	0.76	0.00
X63744	solute carrier family 1 (glial high affinity glutamate transporter), member 3	0.76	0.00
X13016	CD48 Antigen	0.76	0.00
M60654	adrenergic receptor, alpha 1D	0.76	1.72
AF007758	synuclein, alpha	0.78	2.75
X12744	thyroid hormone receptor alpha	0.78	2.75
X04139	protein kinase C, beta	0.78	1.72
NM_053357	catenin (cadherin associated protein), beta 1	0.78	0.00
J04625	carboxypeptidase E	0.79	3.53
E01789	rat C-kinase type-II (beta-2)	0.79	0.00
X62085	hypoxanthine phosphoribosyltransferase 1	0.79	3.53
XM_575489	neurogenic differentiation 6	0.79	0.00
S53987	nACh Receptor, alpha 7 subunit	0.79	5.93
X02231	GAPDH	0.79	3.53
L21192	GAP43	0.80	2.75
AF459021	tubulin, beta 3	0.80	0.00
AF027954	BCL-2 related ovarian killer protein	0.80	0.00
M17069	Calmodulin	0.80	0.00
U08290	Neuronatin	0.80	1.72
M31174	thyroid hormone receptor alpha	0.81	0.00
U17607	nuclear transcription factor-Y gamma	0.81	0.00
X76489	CD9 antigen	0.81	4.36
M25890	Somatostatin	0.81	0.00
S45392	heat shock protein 1, beta, 90 kDa	0.82	5.29
S62933	neurotrophic tyrosine kinase, receptor, type 3	0.83	1.72
S59158	solute carrier family 1 (glial high affinity glutamate transporter), member 3	0.84	4.36
U11031	contactin 3	0.84	3.53
S82649	neuronal pentraxin 2	0.85	3.53
X01032	Cholecystokinin	0.87	8.82
M96376	neurexin 2	0.87	2.75
M64780	Agrin	0.87	5.29
X69903	interleukin 4 receptor, alpha	0.87	6.49

D28561	solute carrier family 2 (facilitated glucose transporter), member 4	0.88	6.49
J05510	IP3 Receptor	0.88	6.49
Y11433	pyrimidinergic receptor P2Y, G-protein coupled, 4	0.88	3.53
Z12152	neurofilament 3, medium	0.88	5.93
D00688	monoamine oxidase A	0.88	7.23
AF020712	large conductance calcium activated potassium channel	0.89	8.82
AF050663	zinc finger binding domain	0.90	5.93
Genes more highly expressed in the right hippocampal formation at P9 following saline injections between P6 and P9			
U92655	potassium voltage-gated channel, subfamily Q, member 1	1.82	0.00
M25157	SOD 1	1.66	0.00
S73424	macrophage migration inhibitory factor	1.51	0.00
X78848	glutathione S-transferase A3	1.46	0.00
D90258	proteasome subunit alpha type 3	1.44	0.00
Y00404	SOD 1	1.42	0.00
X03475	ribosomal protein L35a	1.39	0.00
AF003598	integrin, beta 7	1.37	0.00
M15480	insulin-like growth factor 1	1.37	0.00
S44606	integrin, beta	1.36	0.00
M21060	SOD 1	1.36	0.00
X15013	ribosomal Protein L7a	1.35	1.48
U77776	Interleukin 18	1.29	0.00
X06107	insulin-like growth factor 1	1.28	0.00
S72505	glutathione S-transferase A3	1.28	0.00
D13417	hairy and enhancer of split 1	1.27	1.48
X95096	macrophage stimulating 1	1.26	0.00
AB011679	tubulin, beta 5	1.26	2.60
J05122	benzodiazapine Receptor	1.26	1.48
D38380	signal recognition particle receptor, B subunit; transferrin	1.22	1.48
AF025671	caspase 2	1.22	1.48
AJ012603	A dinintegrin and metalloproteinase domain 17	1.22	2.60
U77777	Interleukin 18	1.20	2.60
AF030253	GABA vesicular transporter, member 1	1.20	5.93
U33472	serine/threonine kinase 10	1.19	3.37
D00698	insulin-like growth factor 1	1.19	1.48
M31837	insulin-like growth factor binding protein 3	1.17	5.93
X86789	synuclein, gamma	1.14	5.93
L36460	interleukin 9	1.14	7.23
SAM paired analysis (FDR<10% at P9 following saline injections) was used to determine the fold change. A fold change greater than 1.0 indicated that the gene was more highly expressed in the right hippocampal formation and a fold change less than 1.0 indicated that the gene was more highly expressed in the left hippocampal formation.			

I utilized DAVID analysis to identify Gene Ontology Terms (GoTerms). The gene ontology (GO) Consortium (available at www.geneontology.org) organized individual

genes into biological pathways within three larger, independent ontological categories based on biological processes, molecular function, and cellular components. The genes identified as significantly differentially expressed in the rat hippocampal formation following saline injections between P6 and P9 using SAM analysis were further analyzed: those findings indicated that 113 GoTerms were identified as being significantly enriched in the left (Appendix C): 10 were in the molecular function category, 21 were in the cellular components category, and 82 were in the biological processes category. Of the 90 GoTerms that were enriched in the right hippocampal formation (Appendix D), 16 were in the molecular function category, 11 were in the cellular components category, and 62 were in the biological processes category.

Following CPP injections between P6 and P9, 27 genes were differentially expressed at P9: 4 genes were more highly expressed in the left and 23 genes were more highly expressed in the right hippocampal formation (Table 6.2). Additionally, 8 GoTerms were enriched in the right hippocampal formation after CPP injections: 4 in the molecular function category, 3 in the cellular components category, and 1 in the biological processes category (Appendix E). No GoTerms were enriched in the left hippocampal formation after CPP injections. Importantly, following CPP injections, the pattern of lateralized gene expression observed during normal development (where the majority of differentially expressed genes were more highly expressed in the left hippocampus) was no longer observed.

Table 6.2: ***Lateralized gene expression in the rat hippocampal formation at postnatal day 9 following CPP injections between P6 and P9***

Gene Bank Accession No.	Gene Name	Fold Change	q-value (%)
Genes more highly expressed in the left hippocampal formation at P9 following CPP injections between P6 and P9			
M83196	microtubule-associated protein 1A	0.82	0.00
J04625	carboxypeptidase E	0.80	0.00
L31622	nACh Receptor, beta 2 subunit	0.85	0.00
AF058795	GABAB Receptor	0.85	0.00
Genes more highly expressed in the right hippocampal formation at P9 following CPP injections between P6 and P9			
D13417	hairy and enhancer of split 1	1.28	0.00
NM_013058	inhibitor of DNA binding 3	1.20	0.00
M18416	early growth response 1	1.19	0.00
X13016	CD48 Antigen	1.19	6.60
V01217	actin, beta	1.18	8.71
Y00396	C-MYC	1.17	0.00
S53987	nACh Receptor, alpha 7 subunit	1.16	8.71
D14048	heterogeneous nuclear ribonucleoprotein U	1.15	6.60
AF022083	G Protein, beta 1	1.14	6.60
D38380	signal recognition particle receptor, B subunit; transferrin	1.14	8.71
M27158	potassium voltage-gated channel	1.13	8.71
AB004267	CAMK	1.13	0.00
AF057308	hypoxia inducible factor 1, alpha subunit	1.13	8.71
M58040	transferrin receptor	1.13	8.71
AB018049	sialyltransferase 9	1.12	8.71
U73142	MAPK 14	1.12	6.60
D84450	ATPase, Na+/K+ transporting, beta 3 polypeptide	1.12	8.71
X06769	C-FOS	1.12	8.71
AJ000556	janus kinase 1	1.11	8.71
M86389	heat shock protein 1, 27 kDa	1.11	8.71
X82021	suppression of tumorigenicity 13	1.11	8.71
M31837	insulin-like growth factor binding protein 3	1.11	8.71
U53859	calpain, small subunit 1	1.09	8.71

SAM paired analysis (FDR<10% at P9 following CPP injections) was used to determine the fold change. A fold change greater than 1.0 indicated that the gene was more highly expressed in the right hippocampal formation and a fold change less than 1.0 indicated that the gene was more highly expressed in the left hippocampal formation.

In order to determine the effect of either a reduction in NMDAR activity or the injection procedure alone, on lateralized gene expression at the end of the first postnatal week, I compared our findings during normal at P6 and P9 (Moskal et al., 2006) to those following CPP, or saline control, injections between P6 and P9 (Rahimi et al., 2006; Gross et al., 2007; Claiborne et al., 2010). Our lab previously found that during normal development all of the differentially expressed genes in the rat hippocampal formation are more highly expressed in the right at P6 (Moskal et al., 2006). Using SAM analysis with a false discovery rate (FDR) of less than 10%, 44 genes were found to be more highly expressed in the right hippocampal formation (Table 6.3).

Table 6.3: ***Genes more highly expressed in the right hippocampal formation at P6***			
Gene Bank Accession No.	**Gene Name**	**Fold Change**	**q-value (%)**
S45392	similar to heat shock protein 1	1.64	0.00
L21192	growth associated protein 43	1.63	0.00
U63740	fasciculation and elongation protein zeta 1 (zygin I)	1.62	0.00
K03486	protein kinase C, beta	1.60	0.00
J04625	carboxypeptidase E	1.53	0.00
V01227	tubulin, alpha	1.52	0.00
D90035	protein phosphatase 3	1.49	0.00
E12625	novel protein expressed with nerve injury	1.44	0.00
U03390	G protein, beta polypeptide 2 like 1	1.44	0.00
X02231	GAPDH	1.40	0.00
D84450	ATPase, Na+/K+ transporting, beta 3 polypeptide	1.35	0.00
D25224	40S ribosomal protein SA	1.34	0.00
X62952	Vimentin	1.33	0.00
M17069	calmodulin	1.32	0.00
U30938	microtubule-associated protein 2	1.30	0.00
AF044581	syntaxin 12	1.29	0.00
U77777	interleukin 18	1.28	0.00
U50194	tripeptidyl peptidase II	1.27	2.26
M86621	calcium channel, voltage-dependent, alpha2/delta subunit 1	1.27	0.00
S59158	solute carrier family 1 (glial high affinity glutamate transporter), member 3	1.27	0.00
X79321	microtubule-associated protein tau	1.25	2.26
S60953	neurotrophic tyrosine kinase, receptor, type 3	1.24	0.00
M96853	discs, large homolog 4	1.21	0.00
AF306546	solute carrier organic anion transporter family, member 1c1	1.21	0.00
M91652	glutamate-ammonia ligase (glutamine synthetase)	1.20	0.00
D28561	solute carrier family 2 (facilitated glucose transporter), member 4	1.20	0.00
AF005099	neuronal pentraxin receptor	1.19	0.00
S73424	macrophage migration inhibitory factor	1.19	2.26
AF061726	calpain 3	1.18	4.09
D30781	Phospholipase A2 receptor	1.17	0.00
M91590	arrestin, beta 2	1.17	7.34
X07467	glucose-6-phosphate dehydrogenase	1.17	2.26
AJ005642	protease, serine, 22	1.16	2.26
M23601	monoamine oxidase B	1.16	2.26
S48813	adrenergic, beta, receptor kinase 1	1.15	2.26
AF089730	potassium channel, subfamily T, member 1	1.15	4.09
U49953	p21 protein (Cdc42/Rac)-activated kinase 1	1.15	8.80
M54987	corticotrophin releasing hormone	1.15	7.34
D12573	Hippocalcin	1.14	7.34
L20822	syntaxin 5	1.14	2.26

S49003	growth hormone receptor	1.13	8.80
AF000423	synaptotagmin XI	1.13	4.09
M17523	peptide YY (mapped)	1.13	4.09
D26154	RB109	1.11	7.34

SAM paired analysis (FDR<10% at P6) was used to determine the fold change. A fold change greater than 1.0 indicated that the gene was more highly expressed in the right hippocampal formation and a fold change less than 1.0 would have indicated that the gene was more highly expressed in the left hippocampal formation. Data from Moskal et al. (2006). An FDR (q-value) of less than 10% was utilized in the present study to compare lateralized gene expression during normal development at P6 and P9 to those following either saline or CPP injections between P6 and P9.

I further examined the 44 genes that were differentially expressed at P6 using DAVID gene functional annotation analysis to identify groups of related genes categorized in gene ontology terms under one of three ontological categories: biological processes, cellular components, or molecular functions. I found that 155 gene ontology terms (GoTerms) were significantly enriched in the right hippocampal formation at P6. More specifically, 95 GoTerms in the biological process, 47 in the cellular component, and 13 in the molecular function gene ontology categories were significantly enriched in in the right hippocampal formation (Appendix F).

In contrast to our lab's previous findings at P6 where all of the differentially expressed genes were more highly expressed in the right hippocampal formation, by P9 the majority of the differentially expressed genes were more highly expressed in the left hippocampal formation at P9 (Moskal et al., 2006). Using SAM analysis with a false discovery rate of less than 10%, 30 genes were differentially expressed in the hippocampal formation of the rat at P9: 22 were more highly expressed in the left and 8 were more highly expressed in the right hippocampal formation (Table 6.4). Thus, our lab previously observed a right-to-left shift in preferential gene expression between P6 and P9 in the rat hippocampal formation.

Table 6.4: *Lateralized gene expression in the rat hippocampal formation at postnatal day 9*

Gene Bank Accession No.	Gene Name	Fold Change	q-value (%)
Genes more highly expressed in the left hippocampal formation at P9 during normal development			
J04218	Glucokinase	0.72	0.00
X06827	hydroxymethylbilane synthase	0.73	0.00
M68971	hexokinase 2	0.74	0.00
AF459021	tubulin, beta 3	0.74	0.00
X15013	Ribosomal Protein L7a	0.75	0.00
AB015946	tubulin, gamma 1	0.75	0.00
X62085	hypoxanthine phosphoribosyltransferase 1	0.76	0.00
AB011679	tubulin, beta 5	0.77	0.00
X66870	lamin A	0.79	0.00
U72353	lamin B1	0.79	3.11
U73859	hexokinase 3 (white cell)	0.80	3.11
NM_012734	hexokinase 1	0.80	0.00
V01227	tubulin, alpha	0.80	6.81
M85035	glutamate receptor, ionotropic, AMPA 2	0.80	2.10
D00698	insulin-like growth factor 1	0.81	0.00
S59158	solute carrier family 1 (glial high affinity glutamate transporter), member 3	0.81	3.11
X02231	GAPDH	0.82	4.20
V01217	actin, beta	0.82	6.00
E12625	novel protein expressed with nerve injury	0.83	0.00
L14323	phospholipase C, beta 1	0.85	2.10
AB003991	synaptosomal-associated protein 25	0.86	9.45
U40395	cannabinoid receptor 1	0.90	6.81
Genes more highly expressed in the right hippocampal formation at P9 during normal development			
X13016	Cd48 molecule	1.44	2.90
X54793	60 kDa heat shock protein	1.34	7.54
AJ222813	interleukin 18	1.28	3.88
M64301	mitogen-activated protein kinase 6	1.26	2.90
D90258	Proteasome	1.22	7.54
D26154	RB109	1.18	9.34
D13985	chloride channel, nucleotide-sensitive, 1A	1.18	3.88
L08497	gamma-aminobutyric acid (GABA) A receptor, gamma 2	1.14	7.54

SAM paired analysis (FDR<10% at P9) was used to determine the fold change. A fold change greater than 1.0 indicated that the gene was more highly expressed in the right hippocampal formation and a fold change less than 1.0 indicated that the gene was more highly expressed in the left hippocampal formation. Data from Moskal et al. (2006). An FDR (q-value) of less than 10% was utilized in the present study to compare lateralized gene expression during normal development at P6 and P9 to those following either saline or CPP injections between P6 and P9.

I further examined the 22 genes that were more highly expressed in the left hippocampal formation at P9 using DAVID gene functional annotation analysis to identify groups of related genes categorized as gene ontology terms under one of three ontological categories: biological processes, cellular components, or molecular functions. I found that 125 GoTerms were significantly enriched in the left hippocampal formation at P9 (Appendix G): 69 GoTerms in the biological processes, 31 in the cellular components, and 25 in the molecular functions. In more closely examining the 8 genes that were more highly expressed in the right hippocampal formation at P9, 16 GoTerms were enriched in the right hippocampal formation at P9 (Appendix H). Of the 16 GoTerms, 14 were in the biological processes category, and 2 were in the molecular function category.

Lateralized gene expression patterns change following saline control and CPP injections between P6 and P9 when compared to normal development at P9.

In order to compare the patterns of lateralized gene expression observed during normal development to those following either saline control or CPP injections, I compared the differential gene expression patterns in each group (Table 6.5). I first determined whether any of the genes that were lateralized at P9 during normal development had the same expression pattern following either saline or CPP injections between P6 and P9. In more closely examining the 30 specific genes that were lateralized in the hippocampal formation during normal development of the rat, I noted that 9 of the 30 genes differentially expressed during normal development at P9 remained more highly expressed in the same hemisphere following saline injections, but those genes were no

longer differentially expressed following a reduction in NMDAR-mediated synaptic activity (Table 6.5, rows 18-26).

Second, I determined whether directional preference in lateralized expression of genes normally differentially expressed at P9 reversed following either saline or CPP injections. Additionally, 1 of the 30 genes, CD48 antigen, was more highly expressed in the right at P9, in the left following saline injections, and in the right following CPP injections (Table 6.5, row 17). Thus, the preferential expression of CD48 antigen expression reversed following saline control, but not CPP injections. One of the 30 genes, beta actin, was not differentially expressed following saline injections, but was more highly expressed in the opposite hemisphere following CPP injections as compared to normal development at P9 (Table 6.5, row 30). Thus, the preferential expression of beta actin reversed following CPP, but not saline control, injections.

Third, I determined whether any of the genes that were normally differentially expressed at P9 were no longer differentially expressed following either saline or CPP injections. Sixteen genes were no longer differentially expressed following either saline or CPP injections between P6 and P9 (Table 5, rows 1-16). Additionally, 3 of the 30 genes, insulin like growth factor, ribosomal protein L7a, and beta5-tubulin were more highly expressed in the opposite hemisphere following saline injections and were not differentially expressed following CPP injections (Table 6.5, rows 27-29).

Table 6.5: *Changes in the differential expression of genes normally lateralized at P9 following either saline or CPP injections between P6 and P9*

Rows	Gene Bank Accession No.	Gene Name	*P9	P9 following saline injections between P6 and P9	P9 following CPP injections between P6 and P9
1	U40395	cannabinoid receptor 1	L	-	-
2	J04218	Glucokinase	L	-	-
3	M85035	glutamate receptor, ionotropic, AMPA 2	L	-	-
4	NM_012734	hexokinase 1	L	-	-
5	U73859	hexokinase 3	L	-	-
6	X06827	hydroxymethylbilane synthase	L	-	-
7	X66870	lamin A	L	-	-
8	U72353	lamin B1	L	-	-
9	L14323	phospholipase C, beta 1	L	-	-
10	AB003991	synaptosomal-associated protein 25	L	-	-
11	E12625	novel protein expressed with nerve injury	L	-	-
12	D13985	chloride channel, nucleotide-sensitive, 1A	R	-	-
13	L08497	GABAA receptor, gamma 2	R	-	-
14	X54793	Heat Shock Protein, 60 kDa	R	-	-
15	M64301	MAPK 6	R	-	-
16	D26154	RB109	R	-	-
17	X13016	CD48 Antigen	R	L	R
18	X02231	GAPDH	L	L	-
19	M68971	hexokinase 2	L	L	-
20	X62085	hypoxanthine phosphoribosyltransferase 1	L	L	-
21	S59158, X63744	solute carrier family 1 (glial high affinity glutamate transporter), member 3	L	L	-
22	V01227	tubulin, alpha 1a	L	L	-
23	AF459021	tubulin, beta 3	L	L	-
24	AB015946	tubulin, gamma 1	L	L	-
25	AJ222813, U77776, U77777	Interleukin 18	R	R	-
26	D90258	proteasome subunit alpha type 3	R	R	-
27	D00698, M15480, X06107	insulin-like growth factor 1	L	R	-
28	X15013	Ribosomal Protein L7a	L	R	-
29	AB011679	tubulin, beta 5	L	R	-
30	V01217	actin, beta	L	-	R

Using SAM paired analysis (FDR<10% at P9 during normal development, and at P9 following saline injections or a reduction in NMDAR mediated synaptic activity). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=6 from 3 litters at P9; n=8 male rats from 4 litters at P9 following saline injection between P6 and P9; n=8 male rats from 4 litters at P9 following a reduction in NMDAR mediated synaptic activity between P6 and P9). * Data from Moskal et al. (2006).

In addition to determining the change in expression for genes that were normally differentially expressed at P9 during normal development, I also examined the additional 53 genes that were differentially expressed following saline injections that were not differentially expressed at P9 during normal development (Table 6.6). Of the 53 genes, 19 were more highly expressed in the right (Table 6.6, rows 1-19) and 34 were more highly expressed in the left (Table 6.6, rows (20-53). Only 4 genes showed the same expression pattern at P9 following CPP injections between P6 and P9 (Table 6.6, rows 1-4). Additionally, 48 of the 53 genes were only differentially expressed following saline injections between P6 and P9 and were not differentially expressed following CPP injections between P6 and P9 (Table 6.6, rows 5-52); thus, the pattern of lateralized gene expression for those 48 genes following a reduction in NMDAR-mediated synaptic activity resembled that seen at P9 during normal development indicating that a reduction in NMDAR-mediated synaptic activity countered the injection effect. One of the 53 genes, the alpha 7 subunit of the nACh receptor, was not differentially expressed during normal development at P9, but was more highly expressed in the left hippocampal formation following saline injections and more highly expressed in the right following CPP injections (Table 6.6, row 53).

Table 6.6: *Genes differentially expressed at P9 following saline injections between P6 and P9*					
Rows	**Gene Bank Accession No.**	**Gene Name**	***P9**	**P9 following saline injections between P6 and P9**	**P9 following CPP injections between P6 and P9**
1	D13417	hairy and enhancer of split 1	-	R	R
2	M31837	insulin-like growth factor binding protein 3	-	R	R
3	D38380	signal recognition particle receptor, B subunit; transferrin	-	R	R
4	J04625	carboxypeptidase E	-	L	L
5	AJ012603	A dinintegrin and metalloproteinase domain 17	-	R	-
6	J05122	benzodiazapine Receptor	-	R	-
7	AF025671	caspase 2	-	R	-
8	S72505, X78848	glutathione S-transferase A3	-	R	-
9	AF003598	integrin, beta 7	-	R	-
10	L36460	interleukin 9	-	R	-
11	S73424	macrophage migration inhibitory factor	-	R	-
12	X95096	Macrophage stimulating 1	-	R	-
13	U92655	potassium voltage-gated channel, subfamily Q, member 1	-	R	-
14	X03475	ribosomal protein L35a	-	R	-
15	U33472	serine/threonine kinase 10	-	R	-
16	M21060, M25157, Y00404	SOD 1	-	R	-
17	AF030253	GABA vesicular transporter, member 1	-	R	-
18	X86789	synuclein, gamma	-	R	-
19	S44606	integrin, beta	-	R	-
20	M60654	adrenergic receptor, alpha 1D	-	L	-
21	M64780	Agrin	-	L	-
22	AF027954	BCL-2 related ovarian killer protein	-	L	-
23	M17069	Calmodulin	-	L	-
24	NM_053357	catenin (cadherin associated protein), beta 1	-	L	-
25	X76489	CD9 antigen	-	L	-
26	X01032	Cholecystokinin	-	L	-
27	U11031	contactin 3	-	L	-
28	D28560	ectonucleotide pyrophosphatase/phosphodiesterase 2	-	L	-
29	L21192, NM_017195	GAP43	-	L	-
30	S45392	Heat Shock Protein 1, beta, 90KDA	-	L	-
31	X69903	interleukin 4 receptor, alpha	-	L	-
32	J05510, U38653	IP3 Receptor	-	L	-
33	D00688	monoamine oxidase A	-	L	-
34	M96376	neurexin 2	-	L	-
35	Z12152	neurofilament 3, medium	-	L	-
36	NM_019218	neurogenic differentiation 1	-	L	-
37	XM_575489	neurogenic differentiation 6	-	L	-

38	S82649	neuronal pentraxin 2	-	L	-
39	U08290	Neuronatin	-	L	-
40	S62933	neurotrophic tyrosine kinase, receptor, type 3	-	L	-
41	U17607	nuclear transcription factor-Y gamma	-	L	-
42	NM_017066	Pleiotrophin	-	L	-
43	AF020712	large conductance calcium activated potassium channel	-	L	-
44	X04139	protein kinase C, beta	-	L	-
45	Y11433	pyrimidinergic receptor P2Y, G-protein coupled, 4	-	L	-
46	U01227	Serotonin Receptor 3A	-	L	-
47	D28561	solute carrier family 2 (facilitated glucose transporter), member 4	-	L	-
48	M25890	Somatostatin	-	L	-
49	AF007758	synuclein, alpha	-	L	-
50	M31174, X12744	thyroid hormone receptor alpha	-	L	-
51	AF050663	zinc finger binding domain	-	L	-
52	E01789	rat C-kinase type-II (beta-2)	-	L	-
53	S53987	nACh Receptor, alpha 7 subunit	-	L	R

Using SAM paired analysis (FDR<10% at P9 during normal development, and at P9 following saline injections). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=6 from 3 litters at P9; n=8 male rats from 4 litters at P9 following saline injections between P6 and P9). * Data from Moskal et al. (2006).

Following a reduction in NMDAR-mediated synaptic activity between P6 and P9, 20 genes were differentially expressed at P9 (Table 6.7). Those 20 genes were not differentially expressed at P9 during normal development, nor were they differentially expressed at P9 following saline injections between P6 and P9. Of the 20 genes that were differentially expressed solely after CPP injections, 17 genes were more highly expressed in the right (Table 6.7, rows 1-17) and 3 were more highly expressed in the left hippocampal formation at P9 (Table 6.7, rows 18-20).

Table 6.7: ***Genes differentially expressed at P9 following CPP injections between P6 and P9***

Rows	Gene Bank Accession No.	Gene Name	*P9	P9 following saline injections between P6 and P9	P9 following CPP injections between P6 and P9
1	D84450	ATPase, Na+/K+ transporting, beta 3 polypeptide	-	-	R
2	U53859	calpain, small subunit 1	-	-	R
3	AB004267	CAMK	-	-	R
4	X06769	C-FOS	-	-	R
5	Y00396	C-MYC	-	-	R
6	M18416	early growth response 1	-	-	R
7	AF022083	G Protein, beta 1	-	-	R
8	M86389	heat Shock Protein 1, 27KDA	-	-	R
9	D14048	heterogeneous nuclear ribonucleoprotein U	-	-	R
10	AF057308	hypoxia inducible factor 1, alpha subunit	-	-	R
11	NM_013058	inhibitor of DNA binding 3	-	-	R
12	AJ000556	Janus kinase 1	-	-	R
13	U73142	MAPK 14	-	-	R
14	M27158	potassium voltage-gated channel	-	-	R
15	AB018049	sialyltransferase 9	-	-	R
16	X82021	suppression of tumorigenicity 13	-	-	R
17	M58040	transferrin receptor	-	-	R
18	AF058795	GABAB Receptor	-	-	L
19	M83196	microtubule-associated protein 1A	-	-	L
20	L31622	nACh Receptor, beta 2 subunit	-	-	L

Using SAM paired analysis (FDR<10% at P9 during normal development, and at P9 following CPP injections). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=6 from 3 litters at P9; n=8 male rats from 4 litters at P9 following CPP injections between P6 and P9). * Data from Moskal et al. (2006).

Saline and CPP injections between P6 and P9 resulted in changes in lateralized expression of structural related genes in the developing rat hippocampal formation. DAVID analysis was further utilized to more closely examine signaling pathways comprised of the specific genes that were differentially expressed at P9 during normal development, or following either saline or CPP injections. This was done in order to identify biological processes that may be influenced by the injection procedure or a reduction in NMDAR-mediated synaptic activity. The pathways identified as having been influenced by either CPP or saline injections between P6 and P9 using DAVID analysis

were structure-related genes (Table 6.8), vesicle trafficking genes (Table 6.9), receptor subunits (Table 6.10), LTP (Table 6.11), calcium signaling (Table 6.12), LTD (Table 6.13), VEGF signaling (Table 6.14), JAK-STAT signaling (Table 6.15), Wnt signaling (Table 16), phosphatidylinositol signaling (Table 6.17), glycolysis (Table 6.18), and MAPK signaling (Table 6.19).

Of the structural-related genes assayed during normal development, all were more highly expressed in the right hippocampal formation at P6 and in the left at P9 (Moskal et al., 2006). Following saline injections between P6 and P9, 4 genes were more highly expressed in the right and 6 were more highly expressed in the left at P9. In contrast, following CPP injections between P6 and P9, 4 genes were more highly expressed in the right hippocampal formation at P9 (Table 6.8). Thus, both the injection procedure itself and a reduction in NMDAR-mediated synaptic activity between P6 and P9 changed the pattern of lateralized expression of genes related to structure.

More specifically, of the 2 genes corresponding to proteins involved in structural development that were more highly expressed in the left hippocampal formation at P9, one of those genes (insulin-like growth factor 1) was more highly expressed in the right hippocampal formation following saline control injections between P6 and P9. Furthermore, beta-actin, which is normally more highly expressed in the left hippocampal formation at P9 is more highly expressed in the right hippocampal formation following CPP injections between P6 and P9.

Table 6.8: ***Differential expression of structure related genes in the rat hippocampal formation***					
Gene Bank Accession No.	**Gene Name**	***P6**	***P9**	**P9 following saline injections between P6 and P9**	**P9 following CPP injections between P6 and P9**
Actin Cytoskeleton					
V01217	actin, beta	-	L	-	R
AF003598	integrin, beta 7	-	-	R	-
U49953	p21 protein (Cdc42/Rac)-activated kinase 1	R	-	-	-
Cell Adhesion					
AF003598	integrin, beta 7	-	-	R	-
M96376	neurexin 2	-	-	L	-
Focal Adhesion					
V01217	actin, beta	-	L	-	R
NM_053357	catenin (cadherin associated protein), beta 1	-	-	L	-
D00698, M15480, X06107	insulin-like growth factor 1	-	L	R	-
AF003598	integrin, beta 7	-	-	R	-
U49953	p21 protein (Cdc42/Rac)-activated kinase 1	R	-	-	-
X04139, K03486	protein kinase C, beta	R	-	L	-
Tight Junction					
V01217	actin, beta	-	L	-	R
NM_053357	catenin (cadherin associated protein), beta 1	-	-	L	-
X04139, K03486	protein kinase C, beta	R	-	L	-
Adherens Junction					
V01217	actin, beta	-	L	-	R
NM_053357	catenin (cadherin associated protein), beta 1	-	-	L	-

Using SAM paired analysis (FDR<10% at E18, P6, P9, at P9 following saline or CPP injections between P6 and P9). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=20 male rats at E18 from 5 litters; n=6 from 3 litters at P6, and P9; n=8 male rats from 4 litters at P9 following saline injection between P6 and P9; n=8 male rats from 4 litters at P9 following a reduction in NMDAR-mediated synaptic activity between P6 and P9). * Data from Moskal et al. (2006); **data from qRT-PCR

Saline and CPP injections between P6 and P9 resulted in changes in lateralized expression of genes related to synaptic activity in the developing rat hippocampal formation.

Synaptic vesicle trafficking genes were no longer differentially expressed at P9 following either saline or CPP injections (Table 6.9). Our lab previously reported that synaptic vesicle trafficking genes were more highly expressed in the right hippocampal formation at P6 and shifted to be more highly expressed in the left hippocampal at P9 (Moskal et al., 2006). In addition to using microarray analysis, Moskal and colleagues (2006) further examined synaptic vesicle trafficking gene expression using qRT-PCR. They examined the expression of 13 additional synaptic vesicle trafficking genes that were not on the original array. They found that 9 of the 10 synaptic vesicle trafficking genes that were differentially expressed at P6 were more highly expressed in the right. In contrast, 5 of 6 differentially expressed genes at P9 were more highly expressed in the left hippocampal formation (Moskal et al., 2006). Thus, synaptic vesicle genes showed a right-to-left shift in synaptic vesicle trafficking gene expression, where the majority of the differentially expressed synaptic vesicle trafficking genes were more highly expressed in the right at P6 and the left at P9.

However, in comparing the microarray results from rats at P6, P9, and at P9 following either saline or CPP injections, I was only able to consider the genes present on the array. Of the 3 synaptic vesicle trafficking genes shown to be differentially expressed using SAM with an FDR of less than 10% at either P6 or P9 none were differentially expressed following either saline or CPP injections between P6 and P9. These findings could indicate that the injections procedure itself resulted in a loss of lateralized vesicle

trafficking gene expression during early postnatal development of the hippocampal formation.

Table 6.9: ***Differential expression of synaptic vesicle trafficking genes in the rat hippocampal formation***

Gene Bank Accession No.	Gene Name	*P6	*P9	P9 following saline injections between P6 and P9	P9 following CPP injections between P6 and P9
AB003991	synaptosomal-associated protein 25	-	L	-	-
AF044581	syntaxin 12	R	-	-	-
L20822	syntaxin 5	R	-	-	-

Using SAM paired analysis (FDR<10% at E18, P6, P9, at P9 following saline or CPP injections between P6 and P9). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=20 male rats at E18 from 5 litters; n=6 from 3 litters at P6, and P9; n=8 male rats from 4 litters at P9 following saline injection between P6 and P9; n=8 male rats from 4 litters at P9 following a reduction in NMDAR-mediated synaptic activity between P6 and P9). * Data from Moskal et al. (2006).

Changes in the expression of genes corresponding to proteins that comprise various receptors were observed following either saline or CPP injections between P6 and P9 (Table 6.10). During normal development, 11 differentially expressed receptor genes were more highly expressed in the right hippocampal formation at P6, whereas by P9, three of 5 differentially expressed receptor genes were more highly expressed in the left hippocampal formation (Table 6.10). Following saline injections between P6 and P9, 11 of 15 differentially expressed receptor genes were more highly expressed in the left hippocampal formation at P9; however, following CPP injections between P6 and P9, five of 7 genes were more highly expressed in the right hippocampal formation at P9 (Table 6.10).

Table 6.10: *Differential expression of receptor subunit genes in the rat hippocampal formation*					
Gene Bank Accession No.	**Gene Name**	***P6**	***P9**	**P9 following saline injections between P6 and P9**	**P9 following CPP injections between P6 and P9**
Neurotransmitters					
U01227	serotonin receptor 3A	-	-	L	-
L31622	nACh Receptor, beta 2 subunit	-	-	-	L
S53987	nACh Receptor, alpha 7 subunit	-	-	L	R
M60654	adrenergic receptor, alpha 1D	-	-	L	-
S48813	adrenergic, beta, receptor kinase 1	R	-	-	-
AF030253	GABA vesicular transporter, member 1	-	-	R	-
AF058795	GABAB Receptor	-	-	-	L
L08497	GABAA receptor, gamma 2	-	R	-	-
J05122	benzodiazapine Receptor	-	-	R	-
M85035	AMPA 2	-	L	-	-
S59158, X63744	solute carrier family 1 (glial high affinity glutamate transporter), member 3	R	L	L	-
Ion					
D13985	chloride channel, nucleotide-sensitive, 1A	-	R	-	-
AF306546	solute carrier organic anion transporter family, member 1c1	R	-	-	-
D84450	ATPase, Na+/K+ transporting, beta 3 polypeptide	R	-	-	R
U53211	amiloride-sensitive cation channel 1	-	-	-	-
AF020712	large conductance calcium activated potassium channel	-	-	L	-
AF089730	potassium channel, subfamily T, member 1	R	-	-	-
M27158	potassium voltage-gated channel	-	-	-	R
U92655	potassium voltage-gated channel, subfamily Q, member 1	-	-	R	-
J05510, U38653	IP3 Receptor	-	-	L	-
M86621	calcium channel, voltage-dependent, alpha2/delta subunit 1	R	-	-	-
U40395	cannabinoid receptor 1	-	L	-	-
Other					
AF005099	neuronal pentraxin receptor	R	-	-	-
D30781	Phospholipase A2 receptor	R	-	-	-
D38380	signal recognition particle receptor, B subunit; transferrin	-	-	R	R
M31174, X12744	thyroid hormone receptor alpha	-	-	L	-
S49003	growth hormone receptor	R	-	-	-
S62933	neurotrophic tyrosine kinase, receptor, type 3	R	-	L	-
X69903	interleukin 4 receptor, alpha	-	-	L	-
Y11433	pyrimidinergic receptor P2Y, G-protein coupled, 4	-	-	L	-
M58040	transferrin receptor	-	-	-	R

D28561	solute carrier family 2 (facilitated glucose transporter), member 4	R	-	L	-

Using SAM paired analysis (FDR<10% at E18, P6, P9, at P9 following saline or CPP injections between P6 and P9). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=20 male rats at E18 from 5 litters; n=6 from 3 litters at P6, and P9; n=8 male rats from 4 litters at P9 following saline injection between P6 and P9; n=8 male rats from 4 litters at P9 following a reduction in NMDAR-mediated synaptic activity between P6 and P9). * Data from Moskal et al. (2006); **data from qRT-PCR

In examining differential gene expression in the long-term potentiation signaling (LTP) pathway during normal development, I found that of the genes differentially expressed at P6 all were more highly expressed in the right hippocampal formation, and of the genes differentially expressed at P9 all were more highly expressed in the left at P9. Interestingly, following saline injections between P6 and P9, preferential gene expression remained higher in the left hippocampal formation at P9 (Table 6.11; Figure 6.1). However, following CPP injections between P6 and P9, genes in the LTP pathway were no longer differentially expressed. Thus, a reduction in NMDAR-mediated synaptic activity resulted in a loss of differential gene expression in the LTP pathway in the developing rat hippocampal formation.

Table 6.11: ***Differential expression of genes in the long-term potentiation signaling pathway in the rat hippocampal formation***

Gene Bank Accession No.	Gene Name	E18	*P6	*P9	P9 following saline injections between P6 and P9	P9 following CPP injections between P6 and P9
M17069	Calmodulin	-	R	-	L	-
M85035	glutamate receptor, ionotropic, AMPA 2	-	-	L	-	-
J05510, U38653	IP3 Receptor	-	-	-	L	-
L14323	phospholipase C, beta 1	-	-	L	-	-
X04139, K03486	protein kinase C, beta	-	R	-	L	-
D90035	protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform	-	R	-	-	-

Using SAM paired analysis (FDR<10% at E18, P6, P9, at P9 following saline or CPP injections between P6 and P9). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=20 male rats at E18 from 5 litters; n=6 from 3 litters at P6, and P9; n=8 male rats from 4 litters at P9 following saline injection between P6 and P9; n=8 male rats from 4 litters at P9 following a reduction in NMDAR-mediated synaptic activity between P6 and P9). * Data from Moskal et al. (2006)

Figure 6.1: ***Lateralized Gene Expression in the Long-Term Potentiation Signaling Pathway.*** Genes differentially expressed at P6 are boxed in grey, those at P9 are boxed in blue, those following saline injections between P6 and P9 are boxed in green, and those following CPP injections between P6 and P9 are boxed in red (see table 6.11). Kegg pathway map (Ogata et al., 1999; http://www.genome.jp/kegg/kegg3a.html)

During normal development, all of the differentially-expressed genes that encode proteins in the calcium signaling pathway were more highly expressed in the right hippocampal formation at P6, and one gene was more highly expressed in the left at P9 (Table 6.12; Figure 6.2). Furthermore, 5 additional genes were more highly expressed in the left at P9 following saline injections between P6 and P9. Thus, saline injections increased the number of genes more highly expressed in the left hippocampal formation as compared to P9. However, the directional preference of the genes differentially expressed at P9 during normal development and following saline injections was the same: the genes were more highly expressed in the left. In contrast, following CPP injections between P6 and P9 the majority of the genes in the calcium signaling pathway were not differentially expressed and the only differentially expressed gene was more highly expressed in the right hippocampal formation. A reduction in NMDAR-mediated synaptic activity resulted in those genes no longer being more highly expressed in the left hippocampal formation following a reduction in NMDAR-mediated synaptic activity.

Table 6.12: ***Differential expression of genes in the calcium signaling pathway in the rat hippocampal formation***

Gene Bank Accession No.	Gene Name	*P6	*P9	P9 following saline injections between P6 and P9	P9 following CPP injections between P6 and P9
Calcium Signaling					
M60654	adrenergic receptor, alpha 1D	-	-	L	-
M17069	Calmodulin	R	-	L	-
S53987	nACh Receptor, alpha 7	-	-	L	R
J05510, U38653	IP3 Receptor	-	-	L	-
L14323	phospholipase C, beta 1	-	L	-	-
X04139, K03486	protein kinase C, beta	R	-	L	-
D90035	protein phosphatase 3	R	-	-	-

Using SAM paired analysis (FDR<10% at E18, P6, P9, at P9 following saline or CPP injections between P6 and P9). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=20 male rats at E18 from 5 litters; n=6 from 3 litters at P6, and P9; n=8 male rats from 4 litters at P9 following saline injection between P6 and P9; n=8 male rats from 4 litters at P9 following a reduction in NMDAR-mediated synaptic activity between P6 and P9). *Data from Moskal et al. (2006)

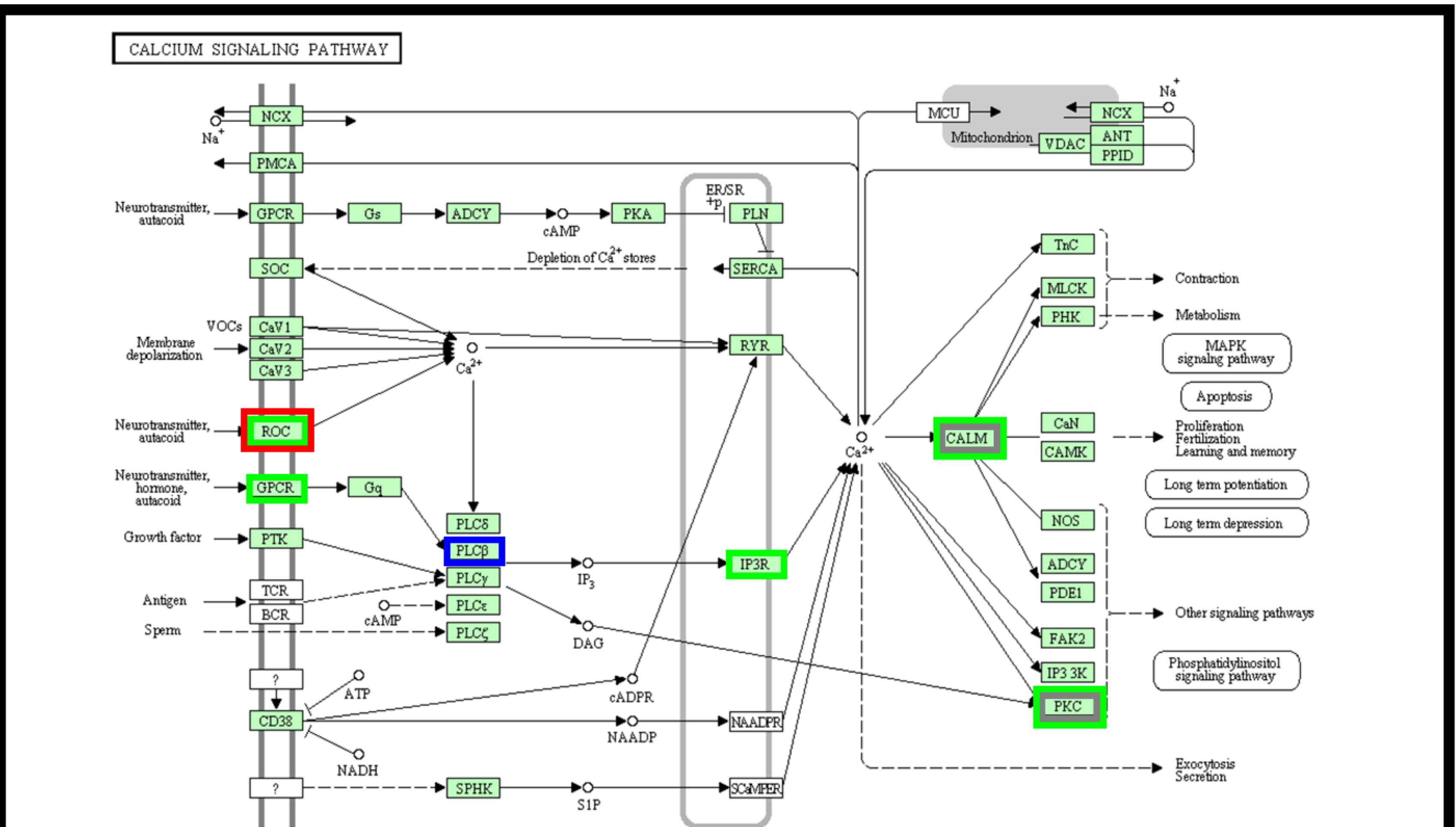

Figure 6.2: ***Lateralized Gene Expression in the Calcium Signaling Pathway.*** Genes differentially expressed at P6 are boxed in grey, those at P9 are boxed in blue, those following saline injections between P6 and P9 are boxed in green, and those following CPP injections between P6 and P9 are boxed in red (see table 6.12). Kegg pathway map (Ogata et al., 1999; http://www.genome.jp/kegg/kegg3a.html)

During normal development, genes encoding for proteins in the long-term depression (LTD) signaling pathway were more highly expressed in the right hippocampal formation at P6 and were more highly expressed in the left hippocampal formation at P9 (Table 6.13; Figure 6.3). Following saline injections between P6 and P9, two genes were more highly expressed in the left hippocampal formation and one gene was more highly expressed in the right hippocampal formation, whereas following CPP injections between P6 and P9, none of the genes was differentially expressed (Table 6.13; Figure 6.3). Thus, genes within the LTD pathway were no longer differentially expressed following a reduction in NMDAR-mediated synaptic activity.

Table 6.13: ***Differential expression of genes in the long-term depression signaling pathway in the rat hippocampal formation***

Gene Bank Accession No.	Gene Name	*P6	*P9	P9 following saline injections between P6 and P9	P9 following CPP injections between P6 and P9
Long-Term Depression					
M54987	corticotrophin releasing hormone	R	-	-	-
M85035	glutamate receptor, ionotropic, AMPA 2	-	L	-	-
J05510, U38653	IP3 Receptor	-	-	L	-
D00698, M15480, X06107	insulin-like growth factor 1	-	L	R	-
L14323	phospholipase C, beta 1	-	L	-	-
X04139, K03486	protein kinase C, beta	R	-	L	-

Using SAM paired analysis (FDR<10% at P6, P9, at P9 following saline or CPP injections between P6 and P9). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=6 from 3 litters at P6, and P9; n=8 male rats from 4 litters at P9 following saline injection between P6 and P9; n=8 male rats from 4 litters at P9 following a reduction in NMDAR-mediated synaptic activity between P6 and P9). * Data from Moskal et al. (2006)

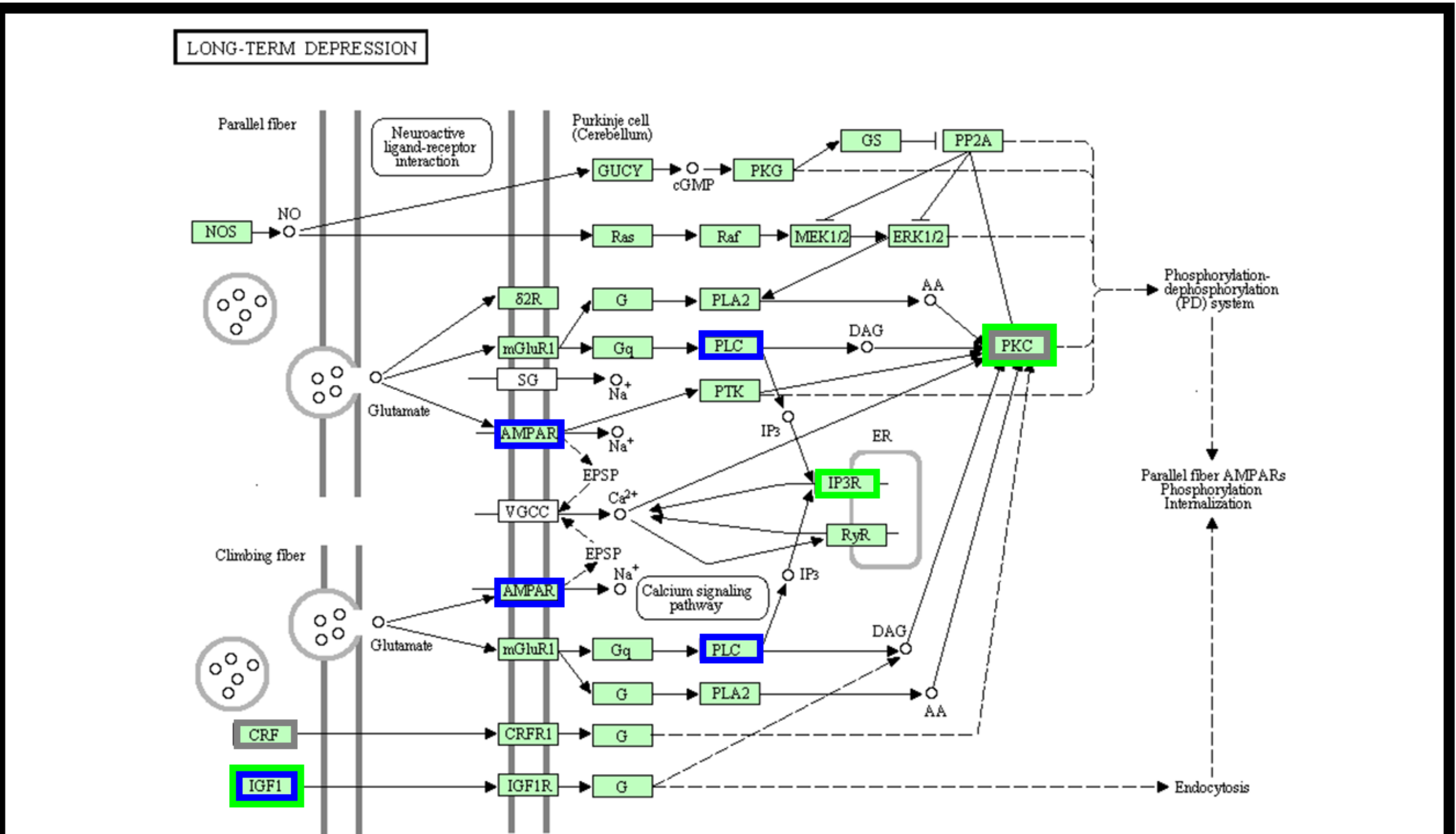

Figure 6.3: ***Lateralized Gene Expression in the Long-Term Depression Signaling Pathway.*** Genes differentially expressed at P6 are boxed in grey, those at P9 are boxed in blue, those following saline injections between P6 and P9 are boxed in green, and those following CPP injections between P6 and P9 are boxed in red (see table 6.13). Kegg pathway map (Ogata et al., 1999; http://www.genome.jp/kegg/kegg3a.html).

Saline and CPP injections between P6 and P9 resulted in changes in lateralized expression of genes related to signaling pathways in the developing rat hippocampal formation.

During normal development, genes corresponding to proteins in the VEGF signaling pathway were more highly expressed in the right hippocampal formation at P6 and were not differentially expressed at P9 during normal development (Table 6.14; Figure 6.4). One gene, PKC, was more highly expressed in the left hippocampal formation at P9 following saline injections between P6 and P9, whereas two genes were more highly expressed in the right hippocampal formation at P9 following CPP injections between P6 and P9. A reduction in NMDAR-mediated synaptic activity resulted in genes within the VEGF signaling pathway being more highly expressed in the right hippocampal formation.

Table 6.14: ***Differential expression of genes in the VEGF signaling pathway in the rat hippocampal formation***

Gene Bank Accession No.	Gene Name	*P6	*P9	P9 following saline injections between P6 and P9	P9 following CPP injections between P6 and P9
VEGF Signaling					
X04139, K03486	protein kinase C, beta	R	-	L	-
D90035	protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform	R	-	-	-
M86389	heat shock protein 1, 27 kDa	-	-	-	R
U73142	MAPK 14	-	-	-	R

Using SAM paired analysis (FDR<10% at P6, P9, at P9 following saline or CPP injections between P6 and P9). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=6 from 3 litters at P6, and P9; n=8 male rats from 4 litters at P9 following saline injection between P6 and P9; n=8 male rats from 4 litters at P9 following a reduction in NMDAR-mediated synaptic activity between P6 and P9).
* Data from Moskal et al. (2006)

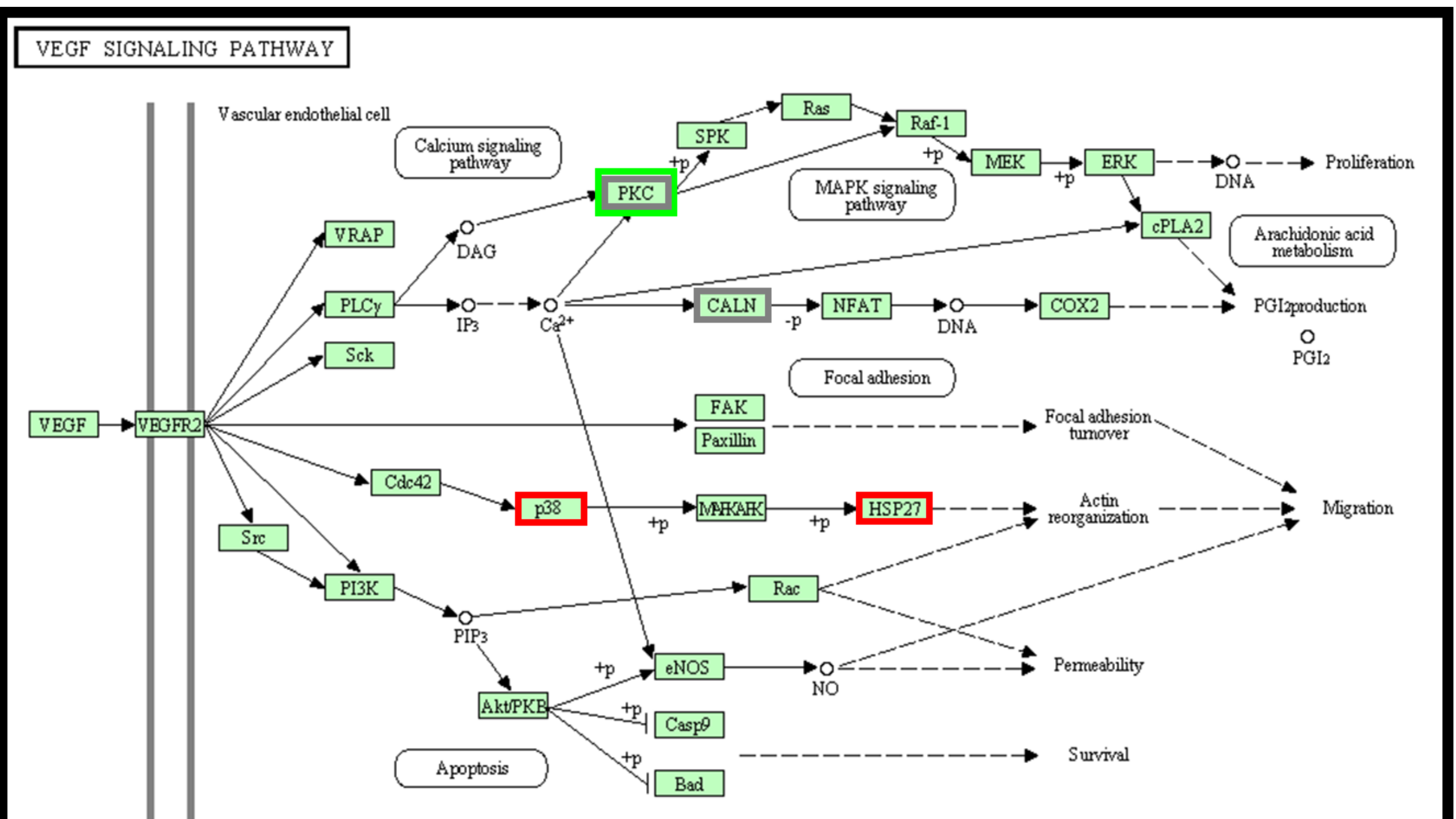

Figure 6.4: ***Lateralized Gene Expression in the VEGF Signaling Pathway.*** Genes differentially expressed at P6 are boxed in grey, those at P9 are boxed in blue, those following saline injections between P6 and P9 are boxed in green, and those following CPP injections between P6 and P9 are boxed in red (see table 6.14). Kegg pathway map (Ogata et al., 1999; http://www.genome.jp/kegg/kegg3a.html).

During normal development, genes corresponding to proteins in the JAK-STAT signaling pathway were more highly expressed in the right hippocampal formation at P6; whereas at P9 lateralization of those genes was no longer observed. Following saline injections between P6 and P9, only two genes were differentially expressed: the alpha subunit of the interleukin 4 receptor was more highly expressed in the left, and interleukin 9 was more highly expressed in the right. In contrast, following CPP injections between P6 and P9, c-myc and Janus kinase 1 were more highly expressed in the right hippocampal formation at P9. Thus, a reduction in NMDAR-mediated synaptic activity between P6 and P9 resulted in genes within the JAK-STAT signaling pathway being more highly expressed in the right hippocampal formation (Table 6.15; Figure 6.5).

Table 6.15: ***Differential expression of genes in the JAK-STAT signaling pathway in the rat hippocampal formation***

Gene Bank Accession No.	Gene Name	*P6	*P9	P9 following saline injections between P6 and P9	P9 following CPP injections between P6 and P9
JAK-STAT Signaling					
S49003	growth hormone receptor	R	-	-	-
Y00396	C-MYC	**R	-	-	R
AJ000556	Janus kinase 1	-	-	-	R
X69903	interleukin 4 receptor, alpha	-	-	L	-
L36460	interleukin 9	-	-	R	-

Using SAM paired analysis (FDR<10% at P6, P9, at P9 following saline or CPP injections between P6 and P9). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=6 from 3 litters at P6, and P9; n=8 male rats from 4 litters at P9 following saline injection between P6 and P9; n=8 male rats from 4 litters at P9 following a reduction in NMDAR-mediated synaptic activity between P6 and P9). * Data from Moskal et al. (2006); **data from qRT-PCR

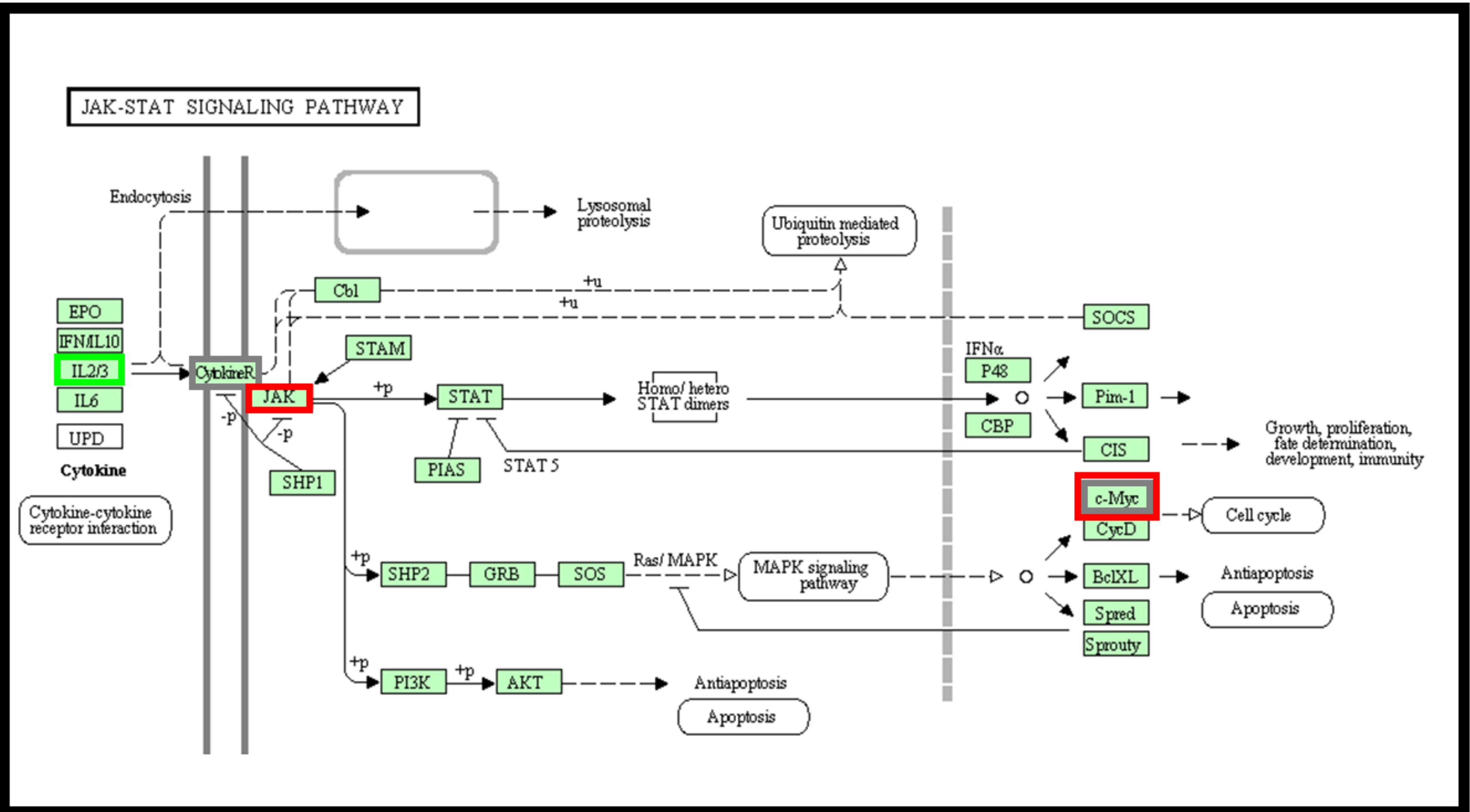

Figure 6.5: ***Lateralized Gene Expression in the JAK-STAT Signaling Pathway.*** Genes differentially expressed at P6 are boxed in grey, those at P9 are boxed in blue, those following saline injections between P6 and P9 are boxed in green, and those following CPP injections between P6 and P9 are boxed in red (see table 6.15). Kegg pathway map (Ogata et al., 1999; http://www.genome.jp/kegg/kegg3a.html).

During normal development, genes corresponding to proteins in the Wnt signaling pathway were more highly expressed in the right hippocampal formation at P6; whereas at P9 one gene, PLC beta 1, was more highly expressed in the left hippocampal formation. Following saline injections between P6 and P9, two genes within the Wnt signaling pathway were more highly expressed in the left hippocampal formation. However, following CPP injections between P6 and P9 only c-myc was more highly expressed in the right hippocampal formation at P9. Thus, a reduction in NMDAR-mediated synaptic activity between P6 and P9 changed the directional preference of lateralized gene expression observed at P9 (Table 6.16; Figure 6.6).

Table 6.16: ***Differential expression of genes in the Wnt signaling pathway in the rat hippocampal formation***

Gene Bank Accession No.	Gene Name	*P6	*P9	P9 following saline injections between P6 and P9	P9 following CPP injections between P6 and P9
Wnt Signaling					
X04139, K03486	protein kinase C, beta	R	-	L	-
D90035	protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform	R	-	-	-
NM_053357	catenin (cadherin associated protein), beta 1	-	-	L	-
Y00396	C-MYC	**R	-	-	R
L14323	phospholipase C, beta 1	-	L	-	-

Using SAM paired analysis (FDR<10% at P6, P9, at P9 following saline or CPP injections between P6 and P9). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=6 from 3 litters at P6, and P9; n=8 male rats from 4 litters at P9 following saline injection between P6 and P9; n=8 male rats from 4 litters at P9 following a reduction in NMDAR-mediated synaptic activity between P6 and P9). * Data from Moskal et al. (2006); **data from qRT-PCR

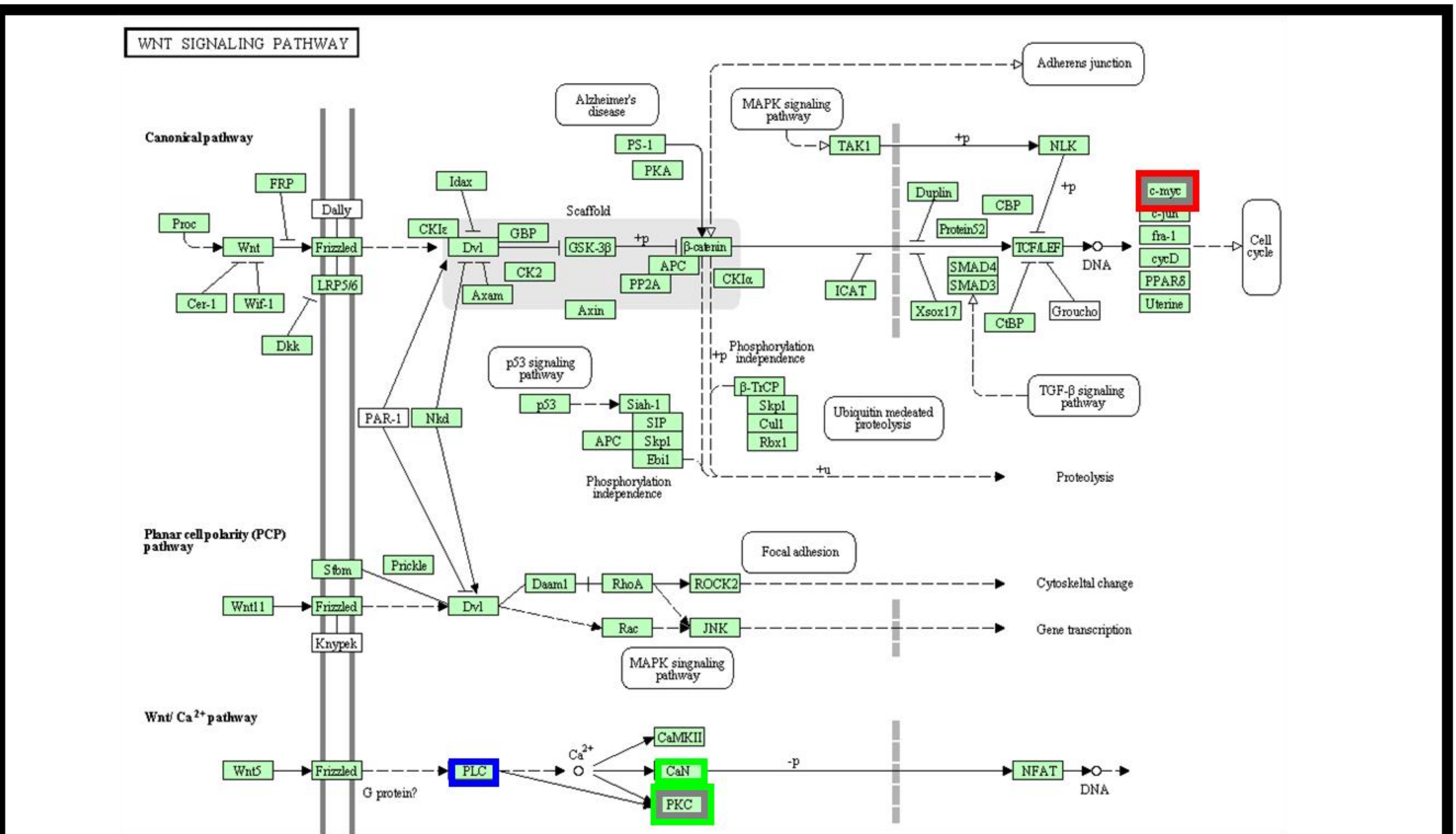

Figure 6.6: ***Lateralized Gene Expression in the Wnt Signaling Pathway.*** Genes differentially expressed at P6 are boxed in grey, those at P9 are boxed in blue, those following saline injections between P6 and P9 are boxed in green, and those following CPP injections between P6 and P9 are boxed in red (see table 6.16). Kegg pathway map (Ogata et al., 1999; http://www.genome.jp/kegg/kegg3a.html).

During normal development, genes corresponding to proteins in the phosphatidylinositol signaling pathway were more highly expressed in the right hippocampus at P6, whereas one gene was more highly expressed in the left at P9 (Moskal et al., 2006; Table 6.17). Following saline injections between P6 and P9, genes in the phosphatidylinositol signaling pathway were also more highly expressed in the left hippocampal formation at P9. In contrast, following CPP injections between P6 and P9 genes within the phosphatidylinositol pathway were no longer differentially expressed. Thus, a reduction in NMDAR-mediated synaptic activity between P6 and P9 results in genes within the phosphatidylinositol pathway being no longer differentially expressed at P9.

Table 6.17: ***Differential expression of genes in the Phosphatidylinositol signaling pathway in the rat hippocampal formation***

Gene Bank Accession No.	Gene Name	P6	*P9	P9 following saline injections between P6 and P9	P9 following CPP injections between P6 and P9
Phosphatidylinositol Signaling					
M17069	Calmodulin	R	-	L	-
J05510, U38653	IP3 Receptor	-	-	L	-
L14323	phospholipase C, beta 1	-	L	-	-
X04139, K03486	protein kinase C, beta	R	-	L	-

Using SAM paired analysis (FDR<10% at P6, P9, at P9 following saline or CPP injections between P6 and P9). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=6 from 3 litters at P6, and P9; n=8 male rats from 4 litters at P9 following saline injection between P6 and P9; n=8 male rats from 4 litters at P9 following a reduction in NMDAR-mediated synaptic activity between P6 and P9). * Data from Moskal et al. (2006); **data from qRT-PCR

Genes corresponding to proteins in the glycolysis signaling pathway were more highly expressed in the right hippocampus at P6 and the left at P9 (Moskal et al., 2006; Table 6.18). Additionally, following saline injections between P6 and P9 genes in the glycolysis signaling pathway were more highly expressed in the left hippocampal formation at P9, whereas following CPP injections between P6 and P9 genes within the glycolysis pathway were no longer differentially expressed. Thus, a reduction in NMDAR-mediated synaptic activity between P6 and P9 resulted in genes within the glycolysis pathway being no longer differentially expressed at P9.

Table 6.18: ***Differential expression of genes in the glycolysis signaling pathway in the rat hippocampal formation***

Gene Bank Accession No.	**Gene Name**	**P6**	***P9**	**P9 following saline injections between P6 and P9**	**P9 following CPP injections between P6 and P9**
Glycolysis					
J04218	Glucokinase	-	L	-	-
NM_012734	hexokinase 1	-	L	-	-
M68971	hexokinase 2	-	L	L	-
U73859	hexokinase 3	-	L	-	-
X02231	GAPDH	R	L	L	-

Using SAM paired analysis (FDR<10% at P6, P9, at P9 following saline or CPP injections between P6 and P9). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=6 from 3 litters at P6, and P9; n=8 male rats from 4 litters at P9 following saline injection between P6 and P9; n=8 male rats from 4 litters at P9 following a reduction in NMDAR-mediated synaptic activity between P6 and P9). * Data from Moskal et al. (2006); **data from qRT-PCR

Genes corresponding to proteins in the MAPK signaling pathway were more highly expressed in the right hippocampal formation at P6 and were not differentially expressed at P9 during normal development, or following saline injections between P6 and P9 (Table 6.19; Figure 6.7). In contrast, following CPP injections between P6 and P9, four genes were more highly expressed in the right hippocampal formation (Table 6.19; Figure 6.7). Thus, a reduction in NMDAR-mediated synaptic activity resulted in genes within the MAPK signaling pathway being more highly expressed in the right hippocampal formation.

Table 6.19: ***Differential expression of genes in the MAPK signaling pathway in the rat hippocampal formation***

Gene Bank Accession No.	Gene Name	*P6	*P9	P9 following saline injections between P6 and P9	P9 following CPP injections between P6 and P9
MAPK Signaling Pathway					
U73142	MAPK 14	-	-	-	R
X06769	C-FOS	-	-	-	R
M86389	heat shock protein 1, 27 kDa	-	-	-	R
Y00396	C-MYC	**R	-	-	R
M91590	arrestin, beta 2	R	-	-	-
M86621	calcium channel, voltage-dependent, alpha2/delta subunit 1	R	-	-	-
X79321	microtubule-associated protein tau	R	-	-	-
U49953	p21 protein (Cdc42/Rac)-activated kinase 1	R	-	-	-
X04139, K03486	protein kinase C, beta	R	-	L	-
D90035	protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform	R	-	-	-

Using SAM paired analysis (FDR<10% P6, P9, at P9 following saline or CPP injections between P6 and P9). R: indicative of higher levels of gene expression in the right hippocampus; L: indicative of higher levels of gene expression in the left hippocampus. (n=6 from 3 litters at P6, and P9; n=8 male rats from 4 litters at P9 following saline injection between P6 and P9; n=8 male rats from 4 litters at P9 following a reduction in NMDAR-mediated synaptic activity between P6 and P9). * Data from Moskal et al. (2006); **data from qRT-PCR

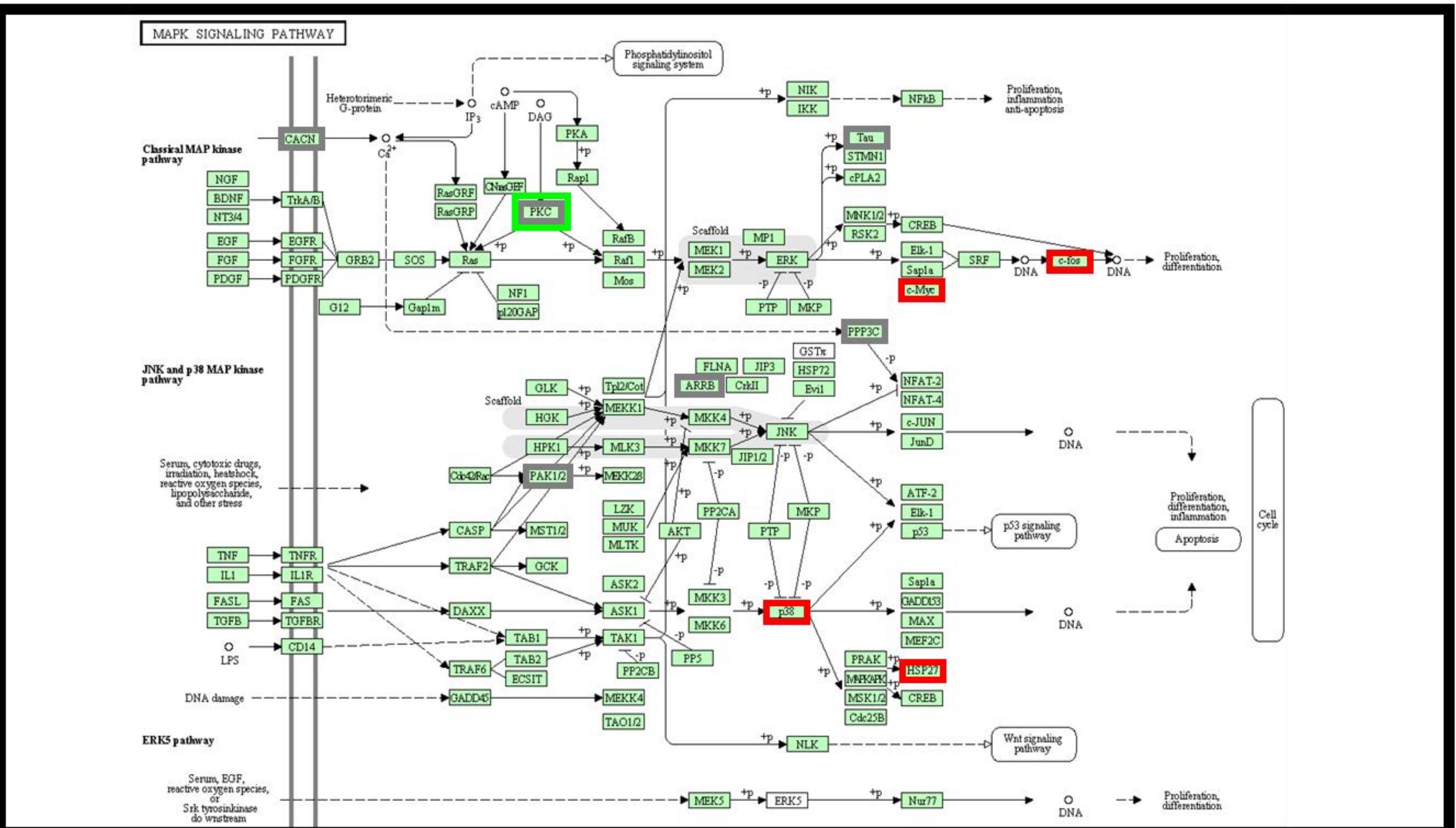

Figure 6.7: ***Lateralized Gene Expression in the MAPK Signaling Pathway.*** Genes differentially expressed at P6 are boxed in grey, those at P9 are boxed in blue, those following saline injections between P6 and P9 are boxed in green, and those following CPP injections between P6 and P9 are boxed in red (see table 6.19). Kegg pathway map (Ogata et al., 1999; http://www.genome.jp/kegg/kegg3a.html).

I utilized qRT-PCR to further examine the expression of genes within the MAPK signaling pathway because the expression of genes within that pathway was clearly affected by a reduction in NMDAR-mediated synaptic activity. Expression of MAPK14 mRNA was not significantly different between the left and right hippocampal formation at postnatal day 6, nor was it greater in the right hippocampal formation as compared to the left in saline-treated rats at P9 (Figure 6.8). MAPK14 mRNA was more highly expressed in the right hippocampal formation (6.87E-10 ± 1.09E-10, mean ± SEM, normalized to cDNA yield) as compared to the left (2.45E-10 ± 7.54E-11; *p = 0.018; Figure 6.8) at P9 following a reduction in NMDAR activity between P6 and P9.

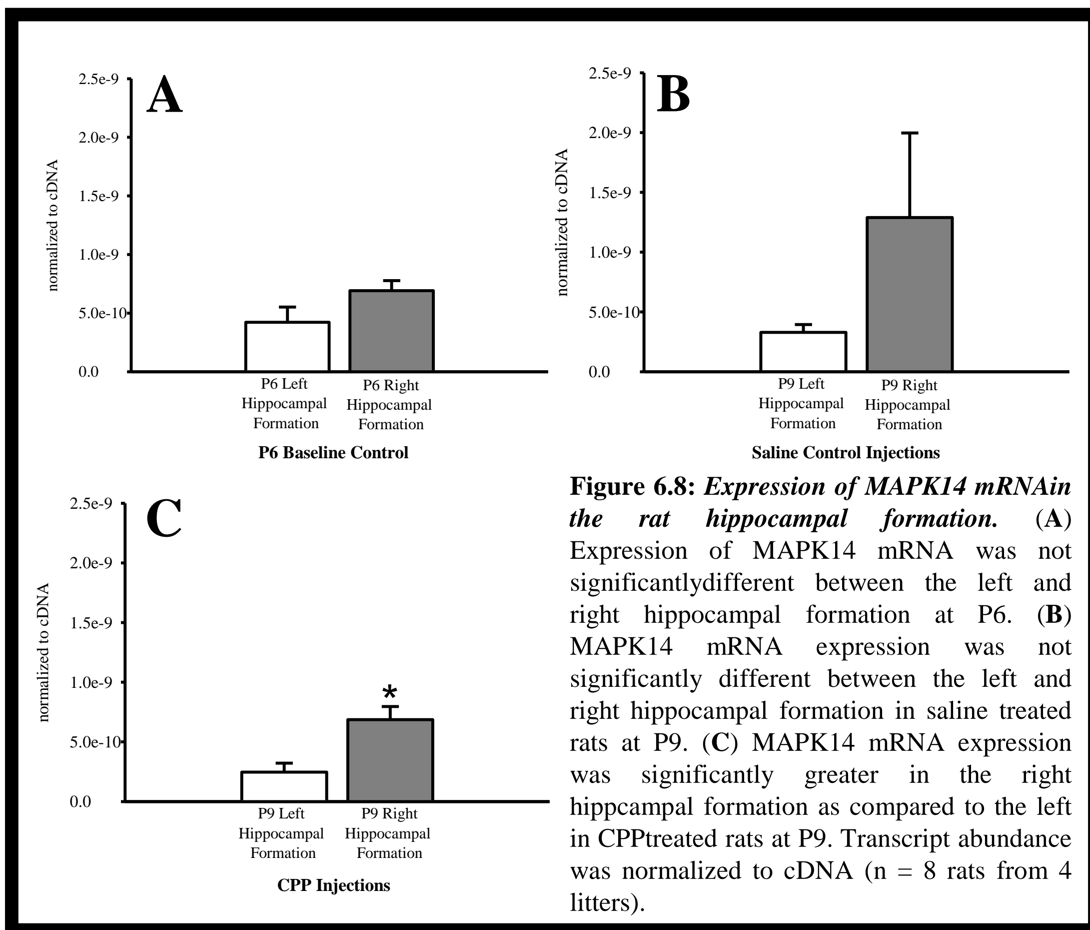

Figure 6.8: ***Expression of MAPK14 mRNAin the rat hippocampal formation.*** (**A**) Expression of MAPK14 mRNA was not significantlydifferent between the left and right hippocampal formation at P6. (**B**) MAPK14 mRNA expression was not significantly different between the left and right hippocampal formation in saline treated rats at P9. (**C**) MAPK14 mRNA expression was significantly greater in the right hippcampal formation as compared to the left in CPPtreated rats at P9. Transcript abundance was normalized to cDNA (n = 8 rats from 4 litters).

c-Myc was more highly expressed in the right hippocampal formation (2.80 E-05 ± 1.12E-5) as compared to the left (8.28 E-07 ± 7.06E-07; *p = 0.044; Figure 6.9A) at P6. The expression of c-Myc was not significantly greater in the right hippocampal formation as compared to the left in saline treated rats (Figure 6.9B). Expression of cMyc mRNA was significantly greater in the right hippocampal formation (1.37E-03 ± 5.31E-04) as compared to the left (4.30E-08 ± 2.14E-08; *p = 0.036; Figure 6.9C) in CPP treated rats. Our data clearly indicate that genes involved in the MAPK signaling pathway were upregulated in the right hippocampal formation during early postnatal development following a reduction in NMDAR-mediated synaptic activity. The microarray and qRT-PCR data indicate that a reduction in NMDAR activity between P6 and P9 resulted in the continued expression of genes in the MAPK signaling pathway in the right hippocampal formation at P9.

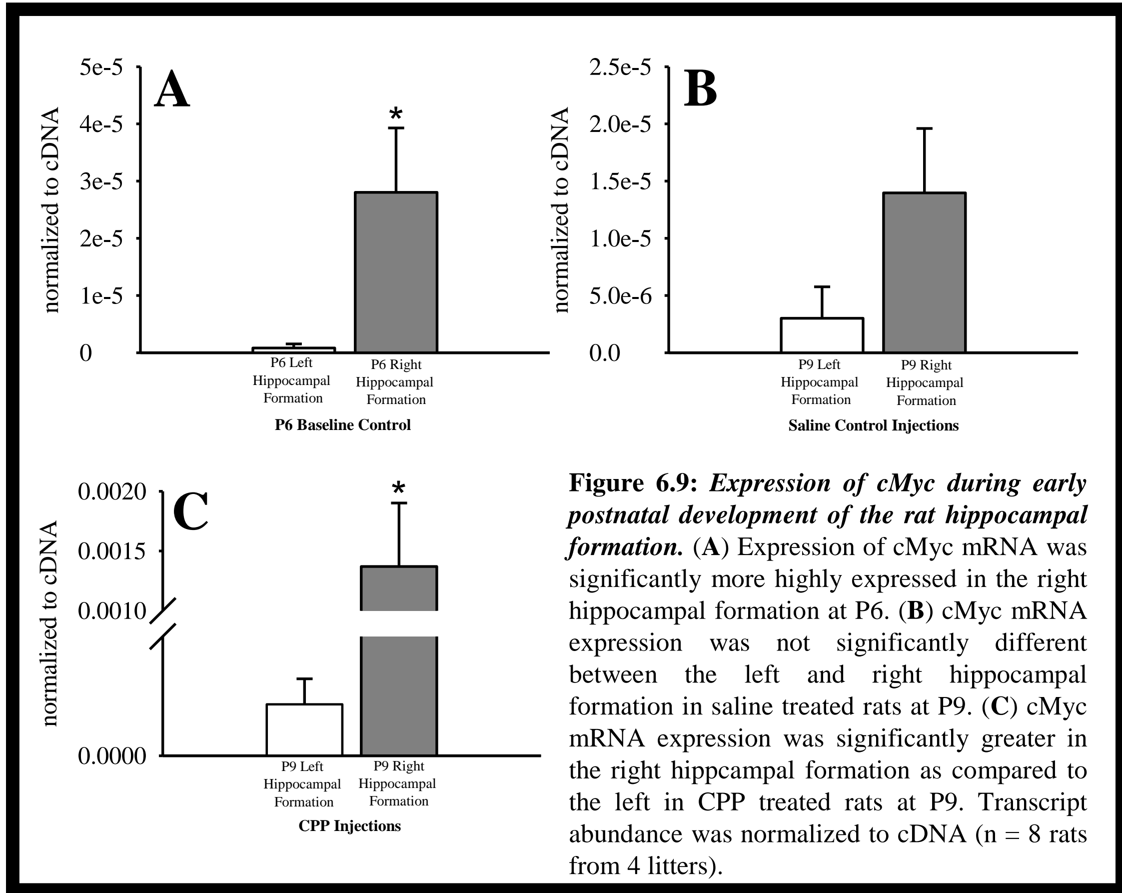

Figure 6.9: ***Expression of cMyc during early postnatal development of the rat hippocampal formation.*** (**A**) Expression of cMyc mRNA was significantly more highly expressed in the right hippocampal formation at P6. (**B**) cMyc mRNA expression was not significantly different between the left and right hippocampal formation in saline treated rats at P9. (**C**) cMyc mRNA expression was significantly greater in the right hippcampal formation as compared to the left in CPP treated rats at P9. Transcript abundance was normalized to cDNA (n = 8 rats from 4 litters).

DISCUSSION

In the present study, gene expression analyses were used to determine the effect of a reduction of NMDAR-mediated synaptic activity between P6 and P9 on lateralized gene expression in the developing rat hippocampal formation. To accomplish this task, I compared the pattern of lateralized gene expression at P6 and P9 during normal development, and at P9 following saline control, or 2mg/kg CPP, an NMDAR antagonist, injections between P6 and P9. A dose of 2 mg/kg CPP was chosen because it was the lowest dose that has been shown to block the induction of long-term potentiation (LTP) on P7 in the rat dentate gyrus (O'Boyle et al., 2004) and this dose of CPP had also been

shown to attenuate the maturation of granule cell neurons when injected between P6 and P9 (Sanchez et al., 2001).

The present findings indicated that, first, the injection procedure between P6 and P9 was sufficient to change lateralized gene expression at P9. Second, data showed that the pattern of lateralized gene expression observed at P9 following a reduction in NMDAR-mediated synaptic activity was unlike the pattern observed during normal development, and, importantly, unlike the pattern observed following saline control injections. Third, I also found that the lateralized expression of structure and development related genes were influenced by the saline injections and a reduction in NMDAR-mediated synaptic activity. Fourth, synaptic vesicle trafficking genes were no longer differentially expressed following the injection procedure. Fifth, it is interesting to note that genes corresponding to receptor proteins involved in hyperpolarization were more highly expressed in the right hippocampal formation at P9 during normal development and following saline injections; however, following CPP injections, these genes were more highly expressed in the left hippocampal formation at P9. Sixth, following CPP injections genes corresponding to proteins in the LTP, LTD, and calcium signaling pathways were no longer differentially expressed in the hippocampal formation at P9, rather than being more highly expressed in the left as was observed during normal development and following saline injections. Lastly, genes corresponding to proteins in the MAPK, Wnt, VEGF, and TGF-Beta signaling pathways were more highly expressed in the right hippocampal formation at P9 following CPP injections between P6 and P9, similar to the pattern normally observed at P6.

Saline-injected control rats were utilized to distinguish the effects of the intraperitoneal injection procedure from the effects of a reduction of NMDAR-mediated synaptic activity. Following saline injections, lateralized gene expression patterns were: i) similar to those observed during normal development at P9; thus, the injection procedure had no effect on the differential expression of those genes; ii) completely lost, thereby resulting in symmetrical gene expression for a particular group of genes; or iii) preferentially expressed in the opposite hemisphere as compared to normal development. Thus, in the second and third instance the injection procedure was sufficient to change the pattern of lateralized gene expression. This could be due to the handling involved with the injection procedure, the stress of the injections, or both.

Previous findings suggest that handling (Denenberg, 1978; Cowell et al., 1997; Tang, 2001, Verstynen et al., 2001; Denenberg, 2005; Tang et al., 2008) and stress (Sullivan and Gratton, 1999; Baum, 2001; Czeh et al., 2008) are both sufficient to influence lateralization during development. Handling alone has been shown to influence lateralization of the brain (Denenberg et al., 1978; Cowell et al., 1997), including the hippocampal formation (Tang, 2001, Verstynen et al., 2001; Tang et al., 2008). Furthermore, handling has been shown to decrease the stress response in the rat hippocampal formation (Meaney et al., 1988; Meaney et al., 1989), and the response to stress has even been shown to be lateralized in other regions of the brain (Sullivan and Gratton, 1998, 1999; Sullivan and Dufresne, 2006).

The idea that changes in lateralized gene expression that occur in the rat hippocampal formation at the end of the first postnatal week following saline injections between P6 and P9 are a result of handling can be supported by the work of Denenberg and

colleagues (Denenberg et al., 1978; Cowell et al., 1997). Denenberg and colleagues showed that handling over the first three postnatal weeks was sufficient to induce left hemisphere dominance in the open field task (Denenberg et al., 1978) and the Morris water-maze task (Cowell et al., 1997). Denenberg et al. (1978) found that when adult rats that were previously handled over the first three postnatal weeks had the right, but not the left, neocortex lesioned, they displayed increased activity in the open-field task. Cowell and colleagues (1997) showed that, when adult rats that were previously handled over the first three postnatal weeks had the right eye blocked, they performed better in the Morris water-maze task as compared to those with the left eye blocked. Importantly these lateralized differences were not observed when rats were not handled over the first three postnatal weeks (Denenberg et al., 1978; Cowell et al., 1997). Thus, Denenberg and colleagues found that some forms of lateralization are observed only when environmental changes occur during early postnatal development.

More specifically, Tang and colleagues (Tang, 2001; Verstynen et al., 2001; Tang et al., 2008) have observed hippocampal lateralization in adult rats, whether handled or not; however, they observed changes in lateralization of the hippocampal formation as a result of handling and novelty exposure in developing rats. Right hippocampal volume was greater in adult rats that were previously handled and exposed to novelty for 3 minutes per day over the first three postnatal weeks, whereas the left hippocampal volume was greater in non-handled rats (Verstynen et al., 2001). Additionally, Tang and colleagues (2008) found that exposure to a novel environment for 3 minutes per day during the first three postnatal weeks resulted in the prolonged maintenance of LTP in the right hippocampus following stimulation of Schaffer collateral axons that synapse on

pyramidal cells in stratum radiatum of CA1 in 7 month old rats, whereas rats not exposed to a novel environment showed no such laterality. Thus, Tang and colleagues have shown (Verstynen et al., 2001; Tang et al., 2008) that some forms of lateralization were observed whether rats are handled or not, but that lateralization of the hippocampal formation in the adult is clearly influenced by environmental changes that occur during early postnatal development.

Previous studies focused on the effect of handling during early development on the hippocampal formation have found that handling influenced the stress response (Meaney et al., 1988; Meaney et al., 1989). Adult rats previously handled over the first three postnatal weeks had higher glucocorticoid receptor densities in the hippocampal formation as compared to non-handled rats, and non-handled rats secreted more glucocorticoids in response to stress (Meaney et al., 1988; Meaney et al., 1989). These changes in the rat hippocampal formation, observed following handling over the first three postnatal weeks, have important functional implications: changes that typically occur as a result of increased glucocorticoid concentrations were attenuated by the handling procedure. Neuronal loss in the hippocampal formation and impairments in the Morris water-maze task in adult rats were much greater in non-handled rats (Meaney et al., 1988; Meaney et al., 1989). Thus, handling has been shown to decrease the effect of stress on the rat hippocampal formation.

It has yet to be determined whether the effects of stress on the rat hippocampal formation are lateralized; however, lateralized responses to stress have been observed in other areas of the rat neocortex (Sullivan and Gratton, 1998, 1999; Sullivan and Dufresne, 2006; Czeh et al., 2008). Sullivan and Gratton (1999) found that a lesion of the

right medial prefrontal cortex (mPFC) lessened gastric ulcer development following cold restraint stress; whereas a lesion of the left mPFC had no effect on stress-induced ulcer development. Additionally, handling has been shown to influence lateralization of the stress response in the infralimbic cortex (Sullivan and Dufresne, 2006). Sullivan and Dufresne (2006) showed that rats handled during early development showed a right shift in dopamine metabolism in the infralimbic cortex following chronic stress in adults, whereas non-handled rats showed increased dopamine metabolism in the left infralimbic cortex following chronic stress. These previous findings indicated that stress effects were lateralized in the rat brain and that the right hemisphere was more involved in the stress response.

Interestingly, Czeh and colleagues (2008) have specifically examined the effect of daily i.p. injections of saline for a period of 21 days, which they defined as a mild stressor that resulted in a preferential reduction in apical dendritic length in the right prelimbic cortex as compared to handled controls. Importantly, these findings indicate the specific stress involved in the i.p. injections of saline has been shown to influence cortical lateralization. The findings in the present study indicate that saline injections between P6 and P9 are sufficient to influence the pattern of lateralized gene expression observed at P9. In considering the previous findings of Czeh and colleagues indicating that the stress of an injection procedure is sufficient to affect anatomical asymmetry in the prelimbic cortex, the changes in lateralized gene expression following saline injections may also be a result of the stress of the injection procedure.

I also examined the effect of CPP injections between P6 and P9 on lateralized gene expression on P9. Following CPP injections, lateralized expression of individual genes

resulted in one of the following possibilities. First, the gene expression pattern was similar to that observed following saline control injections between P6 and P9 – these data would indicate that the gene expression changes were solely a result of the injection procedure. Second, the gene expression pattern was similar to that observed during normal development, but, importantly, not similar to that observed following saline control injections. Third, the gene expression patterns were similar during both normal development and following saline injections between P6 and P9; however, those genes were no longer differentially expressed following CPP injections. In the second and third instances, the particular gene expression pattern detected at P9 following CPP injections between P6 and P9 could be attributed to a result of a reduction in NMDAR-mediated synaptic activity.

Some of the changes in lateralized gene expression that occurred as a result of the saline injections were no longer observed following CPP injections and most closely resembled the gene expression pattern normally observed at P9. In that case, a reduction in NMDAR-mediated synaptic activity reduced at least some of the effect of the injection procedure. This finding is supported by a previous study where a reduction in NMDAR-mediated synaptic activity eliminated the effect of handling-induced changes in gene expression (Garoflos et al., 2007). Garoflos and colleagues (2007) demonstrated that the increased expression of NT-3 in the rat hippocampus that occurs only 4 hours after handling on postnatal day 1 is eliminated following a reduction of NMDAR-mediated synaptic activity using 7mg/kg of CPP. Thus, a reduction in NMDAR-mediated synaptic activity has been previously shown to eliminate the effect of experience during early development.

Importantly, the present findings also indicate that a reduction in NMDAR-mediated synaptic activity had specific effects on lateralized gene expression during early postnatal development that were different from the changes in lateralized gene expression resulting from the saline injection procedure. From these data I concluded that these changes were not a result of the injection procedure and could be attributed to a reduction in NMDAR-mediated synaptic activity. Previous studies, focused on the role of the NMDAR in early development, indicate that the NMDAR plays a significant role in brain development. They clearly suggest that a reduction in NMDAR-mediated synaptic activity in early development has an effect on developing synapses and synaptic activity. During development, hippocampal circuits are refined by activity: the NMDAR is thought to be important in synaptic development by stabilizing synapses that have correlated activity patterns (Scheetz and Constantine-Paton, 1994; Katz and Shatz, 1996; Aamodt and Constantine-Paton, 1999; Minlebaev et al., 2009). Thus, a reduction in NMDAR-mediated synaptic activity in early development was found to interfere with proper synaptic development.

A reduction in NMDAR-mediated synaptic activity has been shown to delay hippocampal development and maturation (Sanchez et al., 2001; Kirov et al., 2004; Kirov et al., 2005; Petrak et al., 2005; Elhart et al., 2010). Importantly, the NMDAR has been shown to modulate neuronal development of the hippocampal formation during the first postnatal week by affecting the maturation of neurons in the dentate gyrus (Sanchez et al., 2001) and the hippocampus proper (Elhart et al., 2010). As I noted in the introduction, Sanchez and colleagues (2001) showed that a reduction of NMDAR-mediated synaptic activity between P6 and P9, using the NMDAR antagonist CPP,

resulted in an increase in the number of immature dentate granule neurons with a corresponding decrease in the number of mature dentate granule neurons. Elhart and colleagues (2010) later showed that following a reduction in NMDAR-mediated synaptic activity between P3 and P17 with MK-801, basal dendrites of CA1 pyramidal cells were shorter and fewer in number, whereas the apical dendrites remained unchanged with a reduction in the number of mature spines on both the apical and basal dendrites. Thus, NMDAR-mediated synaptic activity is likely necessary for the proper maturation of hippocampal neurons. Importantly, these changes in the structure of hippocampal neurons could have important functional implications in the development of hippocampal circuits.

As I stated in the results, I found that the lateralized expression of genes related to structure were influenced by the injection procedure and a reduction in NMDAR-mediated synaptic activity. During normal development all of the differentially expressed genes were more highly expressed in the right hippocampal formation at P6 and in the left hippocampal formation at P9 (Moskal et al., 2006). Following saline injections, only 60% of the differentially expressed genes were more highly expressed in the left hippocampal formation at P9 and following CPP injections all of the differentially expressed genes were more highly expressed in the right hippocampal formation. Taken together, these findings indicated that a reduction in NMDAR-mediated synaptic activity delayed the right-to-left shift in that lateralized expression of structural genes that was normally observed between P6 and P9. These findings are potentially significant as the lateralized expression of those genes could impact the structural development of the hippocampal formation and changes in the lateralized development of the hippocampal formation could potentially impact adult hippocampal function.

To more closely examine the effect of saline injections, CPP injections, or both on the lateralized development of the rat hippocampal formation, I utilized ontological analyses. Although the microarray utilized in the present study did not examine the complete rat genome, the genes present on the array represented more than 90% of the major gene ontological categories (Kroes et al., 2006). Importantly, Significant Analysis of Microarray data (Tusher et al., 2001) generates a ranked list of differentially expressed genes, but does not give any indication of biological mechanisms that might be lateralized during early postnatal development and differentially affected by a reduction in NMDAR-mediated synaptic activity. Subramanian and colleagues (2005) argued that small fold changes in the expression genes within a pathway might be more important that a large fold change in the expression of a single gene. For that reason I also utilized DAVID and GSEA ontological analyses. This allowed for the identification of gene pathways that were significantly differentially enriched in the rat hippocampal formation at P9 during normal development and following saline or CPP injections.

For example, findings in the present study indicate that the expression of genes corresponding to proteins involved in actin remodeling was lateralized during hippocampal development. Genes corresponding to proteins that comprise the actin cytoskeleton were more highly expressed in the right hippocampal formation at P6 and the left hippocampal formation at P9 during normal development (Moskal et al., 2006). In contrast, actin cytoskeleton genes were more highly expressed in the right hippocampal formation following either saline or CPP injections. Thus, actin cytoskeleton genes were preferentially expressed in the right hippocampal formation, rather than the left hippocampal formation at P9 (as was observed during normal development). This change

in preferential expression was likely a result of the injection procedure. Moreover, these data indicate that a left-shift in the preferential expression of actin cytoskeleton genes no longer occurs between P6 and P9 following the injection procedure.

A reduction in actin signaling in the hippocampal formation has been noted following a reduction in NMDAR-mediated synaptic activity (Fischer et al., 2000; Elhart et al., 2010; Medvedev et al., 2010). My findings suggest that the reduction in actin signaling is lateralized and may, at least in part, be a result of the injection procedure alone. Importantly alterations in the expression levels of genes after a reduction in NMDAR-mediated synaptic activity elicit critical changes in the developing brain, including changes in signaling pathways involved in synaptic plasticity, which can ultimately result in changes in neuronal morphology (Elhart et al., 2010). Elhardt and colleagues (2010) have shown that a reduction of NMDAR-mediated synaptic activity between P3 and P17, using the NMDAR antagonist MK-801, affected actin remodeling, protein translation, and hippocampal dependent learning. They found that a reduction in NMDAR-mediated synaptic activity for two weeks during development resulted in a decrease in Rac immunoreactivity, which is involved in actin remodeling, and an increase in m-TOR immunoreactivity, which is involved in protein synthesis, in the mouse hippocampus.

In examining the expression of genes corresponding to proteins that form cell and focal adhesion molecules, it is interesting to note that of the genes that were differentially expressed at P6 during normal development, all were more highly expressed in the right hippocampal formation, whereas at P9 all were more highly expressed in the left hippocampal formation (Moskal et al., 2006). Although genes corresponding to adhesion molecules were differentially expressed following saline injections, half of the

differentially expressed genes were expressed in the left hippocampal formation and half of the differentially expressed genes were expressed in the right hippocampal formation. In contrast, following CPP injections only one gene for an adhesion molecule was more highly expressed in the right hippocampal formation. Thus, following either the injection of saline or CPP, cell and focal adhesion genes were no longer predominantly expressed in the left hippocampal formation.

Cell adhesion molecules (CAM) aid in the formation of connections between neurons during embryonic development (Fields and Itoh, 1996), including the hippocampal formation (Seki and Ruishauser, 1998; Sorra and Harris, 2000). Furthermore, neural activity has been shown to regulate CAM expression, and LTP can be blocked by disrupting CAM expression in the rat hippocampus (Fields and Itoh, 1996). The changes in differential gene expression for cell adhesion molecules that occurred as a result of either saline or CPP injections could be due to changes in synaptic activity.

Our lab previously found that synaptic vesicle trafficking genes were more highly expressed in the right hippocampal formation at P6 and the left hippocampal formation at P9 during normal development (Moskal et al., 2006). Following both saline and CPP injections, synaptic vesicle trafficking genes were no longer differentially expressed. Therefore, the injection procedure eliminated the differential expression of synaptic vesicle trafficking genes. This change in the expression of vesicle trafficking genes could affect synaptic plasticity. If synaptic vesicle recycling was less likely to be the rate limiting step during increased activity in the left hippocampal formation during normal development, then the loss of lateralized expression of those genes could impact hippocampal development.

Two genes corresponding to receptor proteins involved in hyperpolarization, the gamma 2 subunit of the $GABA_A$ receptor and a chloride channel, were more highly expressed in the right hippocampal formation at P9 during normal development. In the rat, GABA initially acts to depolarize neurons and becomes inhibitory with the increased expression of the chloride exporter during postnatal development that leads to a negative shift in the reversal potential for the chloride ions. It is important to note that by P10 $GABA_A$ receptor-mediated hyperpolarization is observed. (Ben-Ari et al., 1994; Gaiarsa et al., 1995; Leinekugel et al., 1999; Ben-Ari, 2001; He et al., 2010). Additionally, the AMPA2 receptor was more highly expressed in the left hippocampal formation, which could contribute to greater depolarization in the left hippocampal formation (Durand et al., 1996; Groc et al., 2002). Taken together, the differential expression of these genes could result in increased activity in the left hippocampal formation as compared to the right at P9 during normal development. Additionally, the genes that were more highly expressed in the right hippocampal formation following saline injections at P9 included the GABA vesicular transporter, the KCNQ1 (Kv7) receptor, and the benzodiazepine receptor. These receptors generally contribute to increased hyperpolarization (Yue and Yaari, 2004; Vervaeke et al., 2006; Mozrzymas et al., 2007; Brown and Passmore, 2009; Leao et al., 2009). Thus, similar to normal development, genes differentially expressed at P9 following saline injections between P6 and P9 indicate increased depolarization in the left hippocampal formation, as compared to the right, at P9.

In contrast to normal development at P9 and saline control injections between P6 and P9, the $GABA_B$ receptor is more highly expressed in the left hippocampal formation following CPP injections, thereby suggesting the possibility that increased inhibition in

the left hippocampal formation (rather than the right) as is observed during normal development at P9 and following saline injections. Moreover, the Na^+/K^+ ATPase (Pellerin and Magistretti, 1996), and the voltage-gated K^+ channel (Grosse et al., 2000), which were more highly expressed in the right hippocampal formation following CPP injections between P6 and P9, are associated with increased synaptic activity. Thus, the changes in the differential expression of genes corresponding to proteins that comprise various receptors could potentially result in greater depolarization in the right hippocampal formation following a reduction in NMDAR-mediated synaptic activity.

Furthermore, the present findings indicate that the lateralized expression of genes involved in synaptic plasticity is observed during early postnatal development and is influenced by NMDAR-mediated synaptic activity. Genes corresponding to proteins involved in calcium signaling, LTP, and LTD were more highly expressed in the left hippocampal formation at P9 during normal development (Moskal et al., 2006). Following saline injections, the directional preference remained the same: of the differentially expressed genes corresponding to proteins in those pathways all were more highly expressed in the left hippocampal formation. In contrast, these genes were no longer differentially expressed at P9 following CPP injections between P6 and P9. These findings are important as they indicate that during normal development, pathways involved in synaptic plasticity are enriched in the left hippocampal formation, whereas following a reduction in NMDAR-mediated synaptic activity those pathways are no longer lateralized.

Genes corresponding to proteins in the MAPK signaling pathway were more highly expressed in the right hippocampal formation at P9 following CPP injections, similar to

the pattern normally observed at P6. During normal development, none of the genes in the MAPK signaling pathway was differentially expressed at P9. In addition, Mitogen-activated protein kinase 14 (MAPK14, also called p38α) and c-Myc were shown to be more highly expressed in the right hippocampal formation following CPP injections using both microarray and qRT-PCR analysis. MAPK activity has been shown to influence the proliferation of stem cells during development (Sato et al., 2008). Thus, the lateralized activation of the MAPK signaling pathway could contribute to the lateralized development of the rat hippocampal formation.

MAPK also has been shown to be upregulated in schizophrenic patients (Kyosseva et al., 1999). Changes in lateralization of the hippocampal formation have been observed in schizophrenic patients (Spaniel et al., 2003; Hanlon et al., 2005; Pilowsky et al., 2006), including changes in NMDAR mediated synaptic activity during hippocampal development (Olney et al., 1999; Bubenikova-Valesova et al 2008; Wedzony et al., 2008). Thus, it is of considerable interest to further characterize the role of the NMDAR in the lateralized development of the rat hippocampal formation.

In summary, my findings indicate that lateralized gene expression in the rat hippocampal formation at the end of the first postnatal week is influenced by the i.p. injection procedure used in the experiment and a reduction in NMDAR-mediated synaptic activity. Upon closer examination, the injection procedure influenced the expression of structural genes, indicating that those genes are influenced by experience during the first postnatal week. Additionally, a reduction in NMDAR-mediated synaptic activity resulted changes in the expression of protein receptor genes corresponding to proteins that could contribute to increased hyperpolarization in the left hippocampal

formation. Furthermore, a loss of lateralized expression for genes corresponding to proteins that comprise the LTP, LTD, and calcium resulted in those genes no longer being more highly expressed in the left hippocampal formation and genes corresponding to proteins in the Wnt, VEGF, and MAPK signaling pathways were more highly expressed in the right hippocampal formation. Taken together, my findings indicate that a reduction in NMDAR-mediated synaptic activity could lead to a delay in the development of the left hippocampal formation.

CHAPTER 7. SUMMARY

The purpose of this dissertation was to characterize lateralized gene expression in the rat hippocampal formation during embryonic and early postnatal development. As shown in Chapter 5, lateralization of gene expression in the hippocampal formation is established during embryonic development. Furthermore, as shown in Chapter 6, lateralization of the hippocampal formation is influenced by early postnatal experience, including the handling and stress associated with the injection procedure and a reduction in NMDAR-mediated synaptic activity at the end of the first postnatal week, which were sufficient to change the pattern of lateralized gene expression observed during early postnatal development. Taken together, these findings are important as they indicate that the establishment of lateralization of the hippocampal formation is genetically controlled, and that lateralization of the hippocampal formation is not merely, or only, "hard-wired" - it is influenced by the changes that occur as a result of one's experience of the environment during early development. In this chapter, I will summarize the results from both Chapters 5 and 6 while focusing on patterns or trends in the data observed during both embryonic and early postnatal development. Furthermore, I will clearly delineate when those patterns are influenced by a reduction in NMDAR-mediated synaptic activity.

Differentially expressed genes were all more highly expressed in the right hippocampal formation at E18

As I discussed in Chapter 5, a directional preference in hippocampal lateralized gene expression is established by E18. All of the differentially expressed genes were more highly expressed in the right hippocampus at E18. Furthermore, as our lab showed

previously (Moskal et al., 2006) and as I reviewed in Chapter 6, the directional preference in lateralized gene expression remains the same during early postnatal development: of the genes differentially expressed at P6 all were more highly expressed in the right hippocampal formation (Moskal et al., 2006). Interestingly, our lab (Moskal et al., 2006) also showed that the majority of genes differentially expressed at P9 were more highly expressed in the left hippocampal formation and continued to be more highly expressed in the young-adult rat at P60. Hence, a right-to-left shift in lateralized gene expression is observed between P6 and P9 and the preferential expression in the left hippocampal formation remains in the adult (Moskal et al., 2006).

Genes shown to be differentially expressed at E18 continue to be differentially expressed at P6 and P9. In more closely examining the 14 genes that were more highly expressed in the right hippocampal formation at E18, 3 of those genes (21%) remained more highly expressed in the right hippocampal formation at P6 (Chapter 5; Table 5.3). Although these data by themselves might not be persuasive enough to argue that lateralized genes remained more highly expressed in the right hippocampal formation at P6, if one considers our lab's previous data (Moskal et al., 2006) where an additional 42 genes (Chapter 6; Table 6.3) were also more highly expressed in the right hippocampal formation at P6, this could indicate that the direction of preferential expression remained in the right hippocampal formation at P6. This could indicate that preferential gene expression is established during embryonic development of the rat hippocampal formation and is preferentially maintained in the right hippocampal formation until at least P6.

Genes preferentially expressed in the right hippocampal formation shift to be more highly expressed in the left hippocampal formation at P9. Of the 7 genes that were differentially expressed at E18 and then later more highly expressed in the left at P9, all of them were more highly expressed in the right hippocampal formation at E18 (Chapter 5; Table 5.3). These findings are intriguing as they suggest that those genes shifted to be more highly expressed in the left hippocampal formation at the end of the first postnatal week and that a directional preference in gene expression displays a right-to-left shift, whereas by P9 the majority of the differentially expressed genes were more highly expressed in the left. It is important to clarify that previous work in our lab (Moskal et al., 2006) indicates an additional 23 genes were also differentially expressed during normal development at P9 (Chapter 6; Table 6.4): 15 (65%) of those genes were more highly expressed in the left and 8 (35%) were more highly expressed in the right hippocampal formation at P9.

Interestingly, 6 of the genes that were differentially expressed at E18 were no longer differentially expressed at either P6 or P9 during normal hippocampal development (Chapter 5; Table 5.3). This indicates that there is the potential for developmental time-point specific lateralized gene expression. The significance of which genes were differentially expressed at specific points in development and the potential implications those findings have on the lateralized development of the rat hippocampal formation will be discussed below.

The right-to-left shift in lateralized gene expression that occurs at the end of the first postnatal week in the rat hippocampal formation is influenced by saline and CPP injections between P6 and P9

In more closely examining the pattern of lateralized gene expression at P9 during normal development and following saline control or CPP injections (Table 6.5), three important observations were made: first, of the genes that had previously been shown to be differentially expressed at P9 (Moskal et al., 2006) with a false discovery rate of less than 10%, the majority (16 of 30 genes - 53%; Table 6.5) were no longer differentially expressed following either saline or CPP injections. Thus, the injection procedure alone was sufficient to change the pattern of lateralized expression for those genes. Second, of the genes differentially expressed at P9, 30% (9 of 30; Table 6.5) showed the same expression pattern following saline injections between P6 and P9. Thus, the saline injections had no effect on the expression of those genes. In contrast, 4 of 30 genes (13%; Table 6.5) were more highly expressed in the opposite hemisphere following saline injections. Thus, the saline injections reversed the preferential expression of those genes in the rat hippocampal formation at P9. Third, 93% (28 of 30 genes; Table 6.5) of the genes normally lateralized at P9 (an additional 40% as compared to the injection procedure alone) were no longer differentially expressed following CPP injections. Thus, these additional changes in lateralized gene expression that did not occur in the saline-injected group are likely a result of a reduction in NMDAR-mediated synaptic activity between P6 and P9.

In addition to changing the pattern of lateralized gene expression normally observed at P9, the saline and CPP injections resulted in unique patterns of lateralized gene

expression. In addition to the 9 genes that were more highly expressed in the same hemisphere at both P9 during normal development and at P9 following saline control injections and the 4 genes that were expressed in the opposite hemisphere (Table 6.5), an additional 53 genes were differentially expressed following saline injections (Table 6.6): 34% in the right hippocampal formation (18 of 53 genes; Chapter 6; Table 6.6) and 66% in the left hippocampal formation (35 of 53 genes; Chapter 6; Table 6.6). Although the specific genes shown to be differentially expressed at P9 following saline injections were not the same as those observed at P9 during normal development, the directional preference of gene expression was similar. The majority of the differentially expressed genes during normal development at P9 were more highly expressed in the left hippocampal formation as compared to the right hippocampal formation (73%; 22 of 30 genes; Table 6.4) and at P9 following saline injections (65%; 43 of 66 genes; Table 6.1).

Only 6% (4 of 87 genes) of the differentially expressed genes in both the saline- and CPP-injected rats had the same directional preference (Table 6.6): 3 in the right hippocampal formation and 1 in the left hippocampal formation at P9 following saline and CPP injections. The change in expression of these genes is likely a result of the injection procedure alone. Interestingly, 3% (2 of 87 genes) were expressed in the opposite hemisphere (Table 6.6) following saline and CPP injections: both were more highly expressed in the left hippocampal formation at P9 following saline injections, but were more highly expressed in the right hippocampal formation CPP injections.

In contrast to normal development and saline control injections, a reduction in NMDAR-mediated synaptic activity resulted in the majority of the differentially expressed genes being more highly expressed in the right hippocampal formation (85%;

23 of 27 genes; Table 6.7) as compared to the left. Although only 3 of the specific genes shown to be differentially expressed following a reduction in NMDAR-mediated synaptic activity were the same as those observed during normal development at P6 (Table 6.7), the directional preference of gene expression was similar between normal animals at P6 and the CPP-injected group. The majority of differentially expressed genes were more highly expressed in the right hippocampal formation at P6 (100%; 45 of 45 genes; Table 6.3) and also at P9 following the reduction in NMDAR-mediated synaptic activity. Thus, in the CPP treated rats, there was not a left-shift in preferential gene expression at P9 as was found in normal development and in the saline-treated group.

Taken together, these findings regarding the directional preference of lateralized gene expression during embryonic and early postnatal development of the hippocampal formation suggest the following conclusion. The right-to-left shift in lateralized gene expression normally observed between P6 and P9 (Moskal et al., 2006) is influenced by early postnatal experience, including a reduction in NMDAR-mediated synaptic activity (Chapter 6). The significance of these findings will be discussed later in Chapter 8.

Differential Expression of Genes Related to Growth and Development during Hippocampal Development

As I discussed in Chapter 5, in more closely examining the specific genes shown to be differentially expressed at E18, those related to cellular growth and development were more highly expressed in the right hippocampal formation. Importantly, these genes continue to be differentially expressed during early postnatal development of the hippocampal formation when the right-to-left shift in lateralized gene expression is

observed (Moskal et al., 2006). Furthermore, saline injections and a reduction in NMDAR-mediated synaptic activity influenced the differential expression of genes related to cellular growth and development. Taken together the data indicate that the saline injections between P6 and P9 resulted in only a partial left-shift in lateralized gene expression in the rat hippocampal formation at the end of the first postnatal week. Additionally, a reduction in NMDAR-mediated synaptic activity between P6 and P9 prevented the left-shift in lateralized expression of cellular growth and development genes in the hippocampal formation that normally occurs between P6 and P9.

More specifically, of the tubulin genes shown to be differentially expressed at E18, all were more highly expressed in the right hippocampal formation (Chapter 5; Table 5.4). By P9, those tubulin genes, and beta actin, were all more highly expressed in the left hippocampal formation. Following saline control injections, 60% (3 of 5 genes; Moskal et al., 2006) of the genes remained more highly expressed in the left hippocampal formation as compared to normal development at P9. In contrast, beta-5 tubulin was more highly expressed in the right hippocampal formation at P9 and beta actin was no longer differentially expressed following saline control injections (Chapter 6). Thus, most of the tubulin structure related genes remained more highly expressed in the left hippocampal formation at P9, thereby indicating that the right-to-left shift in tubulin genes still occurred following saline injections. In contrast, beta-actin was more highly expressed in the right hippocampal formation at P9 following CPP injections and none of the tubulin genes were differentially expressed (Chapter 6). These data indicate that a reduction in NMDAR-mediated synaptic activity prevented the left shift in the expression of genes

important to cell structure (Caceres et al., 1984, 1986; Ambrogini et al., 2004; Dennis et al 2002; Kollins et al., 2009).

Of the genes corresponding to proteins involved in transcription and translation that were differentially expressed at E18, all were more highly expressed in the right hippocampal formation, and one ribosomal protein, 40S ribosomal protein SA, continued to be more highly expressed in the right hippocampal formation at P6 (Chapter 5; Table 5.5; Moskal et al., 2006). By P9, three of the genes were no longer differentially expressed (Moskal et al., 2006) and ribosomal protein L7a was more highly expressed in the left hippocampal formation at P9. In contrast, following saline injections between P6 and P9 the directional preference of lateralized gene expression for genes related to transcription and translation is the same as that observed for E18 and P6 (Moskal et al., 2006; Chapter 6): ribosomal protein L7a and L35a were more highly expressed in the right hippocampal formation. However, following CPP injections between P6 and P9 none of the genes corresponding to proteins involved in transcription and translation was differentially expressed (Moskal et al., 2006; Chapter 6). Thus, the pattern of lateralized gene expression following a reduction in NMDAR-mediated synaptic activity most closely resembled that at P9. Taken together these findings suggest that saline injections between P6 and P9 lead to a pattern that is most similar to that observed at E18 and P6, thereby indicating a delay in development. In contrast, CPP injections between P6 and P9 had almost no effect the differential expression of genes related to transcription and translation as compared to normal development at P9.

Genes corresponding to proteins involved in cellular metabolism and glycolysis are lateralized during hippocampal development (Moskal et al., 2006). At E18, hypoxanthine

phosphoriboxyltransferase 1, a component of the cellular metabolism pathway (Chapter 5; table 5.7) and GAPDH, a component of the glycolysis pathway (Chapter 5; Table 5.8) were more highly expressed in the right hippocampal formation at E18. At P6, all of the differentially expressed genes (4 of 4 genes; Table 5.7 and 5.8) were more highly expressed in the right hippocampal formation. Interestingly, by P9 all of the differentially expressed genes directly related to cellular metabolism (6 of 6 genes; Table 5.7 and 5.8) were more highly expressed in the left hippocampal formation (Moskal et al., 2006). Following saline injections between P6 and P9, 67% (4 of 6 genes) of the differentially expressed genes were more highly expressed in the left hippocampal formation and 33% (2 of 6 genes) were more highly expressed in the right hippocampal formation. Furthermore, none of those genes was differentially expressed following CPP injections between P6 and P9. Therefore, a reduction in NMDAR-mediated synaptic activity resulted in a loss of lateralized expression of genes corresponding to proteins involved in cellular metabolism and glycolysis. Thus, a reduction in NMDAR-mediated synaptic activity was sufficient to change the pattern of lateralized gene expression for genes known to be important in the development of the brain and the establishment of proper neuronal connections (Mody et al., 2001; Loya et al., 2010).

In more closely examining other signaling pathways related to structural growth and development that contained genes shown to be differentially expressed at the end of the first postnatal week (Chapter 6), an interesting trend emerged. Of the genes corresponding to proteins that comprise the actin cytoskeleton, cell adhesion, focal adhesion, tight junction, and adherens junction pathways that were differentially expressed at P6, during normal development all of them were more highly expressed in

the right hippocampus (Moskal et al., 2006). It is important to note that none of the genes within these pathways was differentially expressed at E18. In contrast, by P9, during normal development, all of the genes that were differentially expressed were more highly expressed in the left hippocampus (Moskal et al., 2006). Thus, genes within those pathways showed a right-to-left shift in lateralized gene expression between P6 and P9 during normal development.

Interestingly, following saline or CPP injections the pattern of differential gene expression within the actin cytoskeleton, cell adhesion, focal adhesion, tight junction, and adherens junction pathways changed. Although the specific genes shown to be differentially expressed within these pathways at P9 during normal development were not the same as those following either saline of CPP injections, trends in the directional preference of those genes were still observed. After saline injections between P6 and P9, 40% of the genes were more highly expressed in the right and 60% were more highly expressed in the left hippocampal formation at P9. Thus, in contrast to normal development when all of the differentially expressed structural genes were more highly expressed in the left hippocampal formation (Moskal et al., 2006), 40% of the genes were more highly expressed in the right hippocampal formation following saline injections. Taken together, these data could indicate that the saline injections resulted in a smaller percentage of genes within those pathways shifting to be more highly expressed in the left hippocampal formation. CPP injections between P6 and P9 had an entirely different effect on structural related gene expression at P9 as compared to either normal development or saline injections. As I mentioned in Chapter 6, following CPP injections between P6 and P9, only beta-actin was differentially expressed, and it was more highly

expressed in the right hippocampal formation. Thus, the left-shift that normally occurs between P6 and P9 was lost, thereby suggesting that the shift is at least delayed following a reduction in NMDAR-mediated synaptic activity between P6 and P9. The implications and criticisms of these findings will be discussed in Chapter 8.

Differential Expression of Synapse Genes during Early Postnatal Development of the Rat Hippocampal Formation

In addition to the structural genes that were differentially expressed during hippocampal development, genes related to synaptic plasticity were also differentially expressed at the end of the first postnatal week. It is important to note that none of the vesicle trafficking genes present on the array was differentially expressed at E18 (Chapter 5). As our lab reported previously, the majority of synaptic vesicle trafficking genes were more highly expressed in the right hippocampal formation at P6 and the left hippocampal formation at P9 (Moskal et al., 2006). However, following saline or CPP injections between P6 and P9 none of the synaptic vesicle trafficking genes on the array were differentially expressed (Chapter 6). Thus, the injection procedure alone was sufficient to change the pattern of lateralized synaptic vesicle trafficking gene expression at the end of the first postnatal week.

All of the receptor genes that were differentially expressed at E18 were more highly expressed in the right hippocampal formation (Chapter 5). However this included only 2 of the 32 (6%) receptor genes that were differentially expressed in at least one of the experimental groups at P9. This pattern of differential expression for receptor genes continued until at least P6. By P6, all of the differentially expressed receptor genes were

more highly expressed in the right hippocampal formation (11 of 11 genes; Table 6.10). At P9 a majority (60%; 3 of 5 genes; Table 6.10) of the differentially expressed genes were more highly expressed in the left hippocampal formation, whereas the remaining genes were more highly expressed in the right. Interestingly, upon closer examination, the receptor genes more highly expressed in the right hippocampal formation were both inhibitory receptors: the chloride channel and the $GABA_A$ receptor. In contrast, excitatory receptors were more highly expressed in the left hippocampal formation at P9: AMPA2, CB1 receptor, and the glutamate transporter. These data could indicate that synaptic potentiation is greater in the right hippocampal formation and shifts to be greater in the left by P9 (Moskal et al., 2006).

Following saline injections between P6 and P9, 73% (11 of 15 genes; Table 6.10) of the receptor genes were more highly expressed in the left and 27% were more highly expressed in the right hippocampal formation (Chapter 6). Of the genes that were more highly expressed in the right hippocampal formation following saline injections it is interesting to note that the GABA vesicular transporter, the KCNQ1 (Kv7), and the benzodiazepine receptor gene expression were upregulated in the right hippocampal formation at P9. These receptors have been shown be involved in hyperpolarizing neurons (Yue and Yaari, 2004; Vervaeke et al., 2006; Mozrzymas et al., 2007; Brown and Passmore, 2009; Leao et al., 2009); thus, similar to normal development, genes differentially expressed at P9 following saline injections suggest that synaptic activity may be greater in the left hippocampal formation at P9.

In contrast, following CPP injections between P6 and P9, only 29% (2 of 7 genes; Table 6.10) of the differentially expressed receptor genes were more highly expressed in

the left hippocampal formation and 71% were more highly expressed in the right (Chapter 6). These data suggest that a reduction in NMDAR activity may delay the left shift in lateralized gene expression that normally occurs between P6 and P9. Furthermore, following CPP injections, the $GABA_B$ receptor is more highly expressed in the left hippocampal formation, suggesting that increased hyperpolarization in the left hippocampal formation, rather than the right, as is observed during normal development at P9 and following saline injections. Moreover, the Na^+/K^+ ATPase (Pellerin and Magistretti, 1996), and the voltage-gated K^+ channel (Grosse et al., 2000), which were more highly expressed in the right hippocampal formation following CPP injections are associated with increased synaptic activity. Thus, contrary to normal development and saline control injections, a reduction of NMDAR-mediated may have delayed a left-shift synaptic activity between P6 and P9 in hippocampal activity.

Additionally, genes corresponding to proteins in the calcium signaling, LTP, and LTD pathways were also differentially expressed in the hippocampal formation during postnatal development. Only the alpha7 subunit of the nAChR, a component of the calcium-signaling pathway, was more highly expressed in the right hippocampus at E18 (Chapter 5). At P6, of the genes within those pathways that were differentially expressed, all were more highly expressed in the right hippocampal formation and by P9 all of the differentially expressed genes were more highly expressed in the left (Moskal et al., 2006; Chapter 6). Following saline injections between P6 and P9, 83% (5 of 6 genes; Tables 6.11-6.13) of the differentially expressed genes remained more highly expressed in the left hippocampal formation at P9: the exception being insulin-like growth factor. In contrast, following CPP injections between P6 and P9, the alpha7 subunit of the nAChR

was more highly expressed in the right hippocampal formation and the remaining genes were no longer lateralized (Chapter 6). Thus, a reduction in NMDAR-mediated synaptic activity resulted in a loss of the left-shift in lateralized gene expression in the calcium signaling, LTP, and LTD pathways in the developing rat hippocampal formation.

Taken together, the findings summarized above indicate that synaptic vesicle trafficking genes and genes shown to be involved in synaptic plasticity are differentially expressed at the end of the first postnatal week when synaptic connections are formed during hippocampal development (Tremblay et al., 1988; Durand et al., 1996; Sanchez et al., 2001; O'Boyle et al., 2004; Kirov et al., 2005; Petrak et al., 2005; Moskal et al., 2006). However, CPP injections between P6 and P9 generally resulted in a loss of lateralized gene expression and one gene being more highly expressed in the right hippocampal formation. Thus, a reduction in NMDAR-mediated synaptic activity resulted in a loss of the left-shift in lateralized gene expression normally observed for genes within pathways known to be involved in synaptic plasticity.

Differential Expression of Cell Signaling Pathway Genes during Development of the Rat Hippocampal Formation

In addition to examining signaling pathways specifically involved in plasticity, I further examined signaling pathways with genes that I found to be differentially expressed during early rat hippocampal development. These pathways included Gap Junction, vascular endothelial growth factor (VEGF), janus kinase signal transducer and activator of transcription (JAK-STAT), Wnt, Phosphatidylinositol, and the MAPK

signaling pathway. The implications of the differential expression of genes in these pathways will be discussed later in Chapter 8.

Gap Junction Signaling

As I showed in Chapter 5, genes related to gap junction signaling were more highly expressed in the right hippocampal formation at E18 (Chapter 5). Using microarray analysis, 3 genes (alpha1a-, beta3-, and beta5-tubulin) were found to be more highly expressed in the right hippocampal formation at E18. Alpha1a-tubulin (Figure 6) and Beta3-tubulin (Figure 7) were also shown to be more highly expressed in the right hippocampus at E18 using qRT-PCR analysis. The expression of Connexin43 was also examined at E18 as it is one of the proteins that comprise gap junctions and is thought to be important in the establishment of lateralization (Dermietzel et al., 1989; Bloomstrand et al., 1999; Oviedo and Levin, 2007) and it was also shown to be more highly expressed in the right hippocampus at E18 (Figure 8). Our lab previously showed that the directional pattern of lateralized gene expression is maintained until P6 when alpha1a-tubulin and PKC were shown to be more highly expressed in the right hippocampal formation (Moskal et al., 2006). The directional preference in lateralized gene expression shifted to the left by P9, when 4 genes were shown to be more highly expressed in the left hippocampal formation using microarray analysis (Moskal et al., 2006). Interestingly, following saline injections between P6 and P9, the pattern of lateralized gene expression was similar to that observed during normal development at P9 where the majority of the differentially expressed genes (80%; 4 of 5 genes) were also more highly expressed in the left hippocampal formation. In contrast, following CPP injections between P6 and P9,

genes in the gap junction signaling pathway were no longer differentially expressed at P9, thereby indicating that the left-shift in gene expression did not occur following a reduction in NMDAR-mediated synaptic activity (Chapter 6).

VEGF Signaling

Genes corresponding to proteins in the VEGF signaling pathway were not differentially expressed at 18; rather they were only differentially expressed at the end of the first postnatal week (Table 6.14). Three genes were more highly expressed in the right hippocampal formation at P6, whereas only 1 gene was more highly expressed in the left hippocampal formation at P9 (Moskal et al., 2006). Following saline injections between P6 and P9, the directional preference in lateralized gene expression remained the same as compared to normal development at P9: of the two genes that were differentially expressed at P9, both were more highly expressed in the left hippocampal formation. In contrast to normal development and saline injections, CPP injections between P6 and P9 resulted in heat shock protein 1 and MAPK14 being more highly expressed in the right hippocampal formation at P9.

JAK-STAT Signaling

Genes corresponding to proteins in the JAK-STAT signaling pathway were differentially expressed at P6 (Table 6.15). Growth hormone and c-myc were both more highly expressed in the right hippocampal formation at P6 and by P9 none of the genes in the JAK-STAT signaling pathway were differentially expressed during normal development (Moskal et al., 2006). In contrast to normal development at P9, following

CPP injections c-myc and janus kinase 1 were more highly expressed in the right hippocampal formation at P9.

Wnt Signaling

Genes corresponding to proteins in the Wnt signaling pathway were differentially expressed in the hippocampal formation at the end of the first postnatal week (Table 6.16). Genes in the Wnt signaling pathway were not differentially expressed at E18; however by P6 all (3 of 3) of the differentially expressed genes were more highly expressed in the right hippocampal formation at P6 (Moskal et al., 2006). At P9 only 1 gene, PLC, was more highly expressed in the left hippocampal formation (Moskal et al., 2006). All of the genes (2 of 2 genes) differentially expressed at P9 following saline injections between P6 and P9 were more highly expressed in the left hippocampal formation. In contrast, only one gene (c-myc) was differentially expressed at P9 following CPP injections between P6 and P9, and it was more highly expressed in the right hippocampal formation.

Phosphatidylinositol Signaling

Genes corresponding to proteins in the Phosphatidylinositol signaling pathway were differentially expressed at P6 and P9 during normal development (Table 6.17). At P6 all of the differentially expressed genes were more highly expressed in the right hippocampus whereas at P9, PLC was more highly expressed in the left hippocampal formation (Moskal et al., 2006). Following saline injections, all of the differentially expressed genes were more highly expressed in the left hippocampal formation. In

contrast, following CPP injections between P6 and P9 none of the genes in the phosphatidylinositol signaling pathway were differentially expressed.

MAPK Signaling

Genes corresponding to proteins in the MAPK signaling pathway were not differentially expressed at E18; however, they were more highly expressed in the right hippocampal formation at P6 (Table 6.19). Of the 7 genes that were differentially expressed at P6, all were more highly expressed in the right hippocampal formation (Moskal et al., 2006). However, during normal development, none of the genes in the MAPK signaling pathway was differentially expressed at P9. Following saline injections between P6 and P9, only 1 gene, PKC, was differentially expressed at P9. In contrast to normal development and the saline-injected group, all of the differentially expressed genes (4 of 4 genes) were more highly expressed in the right hippocampal formation following CPP injections. Thus, following a reduction in NMDAR-mediated synaptic activity between P6 and P9, the genes were more highly expressed in the right hippocampal formation at P9. Therefore, the directional pattern of gene expression was similar at P6 and at P9 following a reduction in NMDAR-mediated synaptic activity.

Interestingly, the protein kinase C (PKC) gene is an integral component of many of the pathways discussed above. PKC is a component of the gap junction, VEGF, Wnt, Phosphatidylinositol, and MAPK signaling pathways. PKC is activated by a variety of signaling mechanisms, including calcium and diacylglycerol, to control the function of other proteins through phosphorylation (Purves et al., 2004). The upregulation of PKC expression specifically and the further enrichment of genes within those pathways in the

right hippocampal formation indicate that the further examination of lateralized PKC expression specifically during hippocampal development and in the adult rat might be of particular interest.

CHAPTER 8. DISCUSSION

My findings suggest that the potential for a lateralized rate of hippocampal cell proliferation and maturation established in embryonic development and continuing in early postnatal development leads to early development of the right hippocampus. This could contribute to lateralized synaptic activity, which leads to the differential activation of signaling pathways in the hippocampal formation that have potentially significant effects on the continued development of hippocampal circuitry and function. Importantly, our lab observed a right-to-left shift in lateralized gene expression between P6 and P9, and in this dissertation I presented results indicating that early postnatal experience, including saline injections and Hebbian synaptic action by a reduction in NMDAR activity, delays the left-shift in gene expression. A delay in the left-shift in lateralized gene expression could have potentially significant effects on the development of hippocampal circuitry and later hippocampal function. In the following chapter I will discuss the lateralized gene expression patterns observed in the developing rat hippocampal formation while focusing on the potential implications of those gene expression patterns on cell proliferation and maturation, synaptic activity, and differential activation of signaling pathways, and suggesting future experiments to investigate the implications of my findings. Lastly, I will discuss the implications of my findings on hippocampal function.

Possible Lateralized Cell Proliferation and Maturation in the Embryonic Hippocampal Formation

Genes important to cellular growth and development were more highly expressed in the right hippocampal formation at E18. They continued to be more highly expressed in the right hippocampal formation at P6, and they shifted to be more highly expressed in the left at P9. These findings suggest that cell proliferation was greater in the right hippocampus at E18 and then shifted to be greater in the left hippocampal formation during normal development at P9. To examine the possibility that the rate of neurogenesis is lateralized hippocampal development, I would measure the rate of neurogenesis in the rat hippocampus at E18, P6, and P9 using BrdU incorporation. I expect that neurogenesis would be greater in the right hippocampus at E18 and P6, and greater in the left hippocampal formation at P9 during normal development.

Interestingly, I found that a reduction in NMDAR-mediated synaptic activity between P6 and P9 resulted in a loss of the lateralized expression of cellular growth and development genes. An important implication of these data is that a left-shift in the rate of hippocampal neurogenesis could be delayed, or might no longer occur following a reduction in NMDAR-mediated synaptic activity. To determine whether CPP injections would affect lateralized hippocampal neurogenesis I would compare the rates of neurogenesis in rats during normal development to in three groups of rats, i) rats injected with CPP beginning at P6 and BrdU ii) rats injected with saline beginning at P6 and BrdU, and, iii) rats only given BrdU injections. The BrdU injections would be given once on the morning of P6, P7, P8, or P9 and the rats would be sacrificed 12 hours later to count the number of cells actively undergoing neurogenesis in the left and right

hippocampal formation of each group. I would expect that control rats only injected with BrdU or those rats injected with saline and BrdU would show greater rates of neurogenesis in the left hippocampal formation by P9. In contrast, I anticipate that by P9 the rate of neurogenesis in the rat hippocampal formation would no longer be lateralized following CPP injections between P6 and P9.

In the present study, I examined the lateralized expression of genes within the entire hippocampal formation. As subregion-specific effects have been observed in studies examining total cell number in adult rats, it would be of particular interest to determine whether the potential lateralization of neurogenesis during hippocampal development is subregion specific. For this reason, I would also specifically count the cells in the left and right CA1, CA3, and the dentate gyrus.

Importantly, lateralized rates of neurogenesis could contribute to volumetric differences that have been previously observed. I would also expect that the hippocampal volume would be greater in the right hippocampus at E18. Previous findings indicated that left hippocampal volume was greater in the adult rat and the pattern of lateralized gene expression during normal development at P9 indicate that this volumetric difference may be observed as early as the end of the first postnatal week. To examine this possibility, I would measure hippocampal volume at E18 and P9 and I expect that hippocampal volume would be greater in the right hippocampus at E18 and the left hippocampal formation at P9.

A reduction in NMDAR-mediated synaptic activity could also influence lateralized hippocampal volume. Previous findings suggest that changes in the environment that include handling and novelty exposure during early postnatal development eliminate a

left-shift in hippocampal volume, resulting in a loss of volumetric asymmetry in the adult rat. Previous studies also suggest that novelty effects require the activation of the NMDAR. My data are seemingly in contrast to these findings as they suggest that a reduction in NMDAR-mediated synaptic activity might also prevent a left shift in hippocampal volume that could occur between P6 and P9. It would be of interest to conclusively determine whether CPP injections between P6 and P9 would result in a loss of hippocampal asymmetry as early as P9 and whether the loss would be maintained in the adult rat. Those findings would suggest that a left-shift in volumetric asymmetry is at least partially dependent upon the NMDAR.

Alternatively, there may be no lateralized rates of neurogenesis or hippocampal volume during hippocampal development. This would not preclude the possibility that cells within the right hippocampal formation still mature before those in the left. In that instance the right-to-left shift in the lateralized expression of genes involved in cell proliferation and maturation would indicate that rather than a greater number of neurons in one hemisphere or the other, that cells mature in the right hippocampal formation first. Additionally, a reduction in NMDAR-mediated synaptic activity might influence the lateralized growth and maturation of those cells. Our lab previously showed that CPP injections between P6 and P9 resulted in a greater proportion of immature dentate granule neurons as compared to normal development at P9. Others have shown that a reduction in NMDAR-mediated synaptic activity results in longer more immature spines. Taken together with my findings these data suggest that a reduction in NMDAR-mediated synaptic activity might delay a left-shift in hippocampal maturation. To test these alternative hypotheses, I would evaluate the presence of mature features on dentate

granule neurons and hippocampal pyramidal neurons at P6, P9, and following either saline or CPP injections between P6 and P9. To accurately assess the maturation of those cells I would focus on the oldest cells within the dentate (granule cells in the suprapyramidal blade) and in the hippocampus proper (CA3b) in the left and right hippocampal formation at P6 and P9. I would expect that at P6 a greater percentage of cells within the right hippocampal formation would show mature features as compared to the left, whereas at P9 a greater percentage of cells within the left hippocampal formation would show mature features in the dentate and hippocampus proper. I also expect that, similar to normal development, following saline injections between P6 and P9 a greater percentage of mature cells would be observed in the left hippocampal formation at P9. Following CPP injections between P6 and P9, I anticipate that both the left and right hippocampal formation would have an equal proportion of immature neurons.

If, as I suggested above, cells within the right hippocampal formation are born and mature first, then they might also form functional synaptic connections first. More specifically, cells within the right hippocampus might be more likely to comprise the hippocampal commissure during early development as they would grow and mature to form contralateral projections before the left. Commissural projections are not observed in the rat hippocampal formation until after birth and near the end of the first postnatal week. Interestingly, this coincides with the left shift in lateralized gene expression normally observed at P9. To test this possibility, I would label neurons in either the left or right hippocampal formation at P6. I expect that cells labeled in the right hippocampal formation would account for a greater percentage of the commissural axons when compared to those labeled in the left. If the earlier development of the right hippocampal

formation leads to asymmetric afferent input across the hippocampal commissure, then the synaptic input from the early developing right hemisphere might drive the development of the left hemisphere. This would have important implications to the development of hippocampal circuitry and might also account for any left-shift in hippocampal development that occurs between P6 and P9.

Possible Lateralized Synaptic Activity in the Rat Hippocampal Formation

The potential for the delayed development of the left hippocampal formation discussed above also has important implications for the formation of hippocampal circuitry. It has been argued that structures within the brain that develop more slowly show greater functional plasticity. I also observed lateralized gene expression patterns at the end of the first postnatal week suggesting that vesicular release of neurotransmitter and potentiation could be greater in the right hippocampal formation at P6 and shift to be greater in the left by P9. Similar to normal development at P9, genes differentially expressed at P9 following saline injections between P6 and P9 indicated the possibility that synaptic plasticity was greater in the left hippocampal formation at P9. In contrast to normal development and saline injections, a reduction of NMDAR-mediated synaptic activity between P6 and P9 appeared to have delayed this left-shift in hippocampal plasticity.

The preferential expression of synaptic vesicle trafficking genes in the right hippocampal formation at P6 and the left hippocampal formation at P9 suggests that vesicle priming, fusion, and recycling might also be lateralized. Three possibilities could account for the increased expression of vesicle trafficking genes: i) the readily releasable

pool is larger, ii) the reverse pool of vesicles is larger, or iii) vesicles are recycled at faster rates in the right at P6 and the left hippocampal formation at P9.

Electron microscopy of synapses in the hippocampal formation would permit counting the number of vesicles in the readily releasable and reserve pool of vesicles in the left and right hippocampal formation to determine if any significant lateralization is observed. The readily releasable pool is distinguished from the reserve pool based on the location in the synapse – the readily releasable pool is docked to the cell membrane. To examine the possibility of an increased rate in vesicle recycling in the right hippocampal formation at P6 and the left hippocampal formation at P9, I would utilize an FM dye, such as FM1-34. Once FM dyes bind to cellular membranes they fluoresce; thus, an increase in fluorescence would be correlated with an increase in the uptake of the FM1-34 dye. If vesicle recycling occurs at a faster rate in one hemisphere, then fluorescence would be greater. I expect that vesicle recycling would be greater in the right hippocampal formation at P6 and the left at P9.

Moreover, the Glial high-affinity glutamate transporter was more highly expressed in the left hippocampal formation at P9 during normal development. Thus, in addition to the increase in synaptic vesicle trafficking gene expression in the left hippocampal formation at P9 these data suggest that the rate of glutamate removal is greater in the left hippocampal formation at P9 during normal development. This would keep glutamate at lower levels in the synapse, which would reduce the possibility of NMDAR activation and subsequent excitotoxicity by which high levels of glutamate can kill neurons. Glial glutamate transporters also allow glutamate to be recycled for later use by the presynaptic cells by storing glutamine in vesicles which are released by glia and transported back into

the presynaptic neuron to be converted back to glutamate and stored in vesicles. A similar increase in glutamate transporter expression in the left hippocampal formation was also observed following saline injections.

The GABA vesicular transporter was more highly expressed in the right hippocampal formation following saline injections between P6 and P9. The differential expression of this gene following saline injections between P6 and P9 would be expected to relate to an increase in vesicular GABA content which could result in an increase in the quantal size even without lateralization of synaptic vesicle trafficking. This possibility could be assessed by measuring miniature postsynaptic potential (mIPSP) size. I would expect that mIPSP size is greater in the right hippocampal formation at P9 following saline injections.

As I outlined in previous chapters, there is the potential for increased long-term synaptic plasticity in the right hippocampal formation at P6 and the left hippocampal formation at P9 during normal development and following saline injections. Importantly, this implies that there might be a left-shift in synaptic plasticity between P6 and P9. LTP and LTD are both considered to be models of the cellular processes that underlie learning and memory, and therefore, are considered extremely important in the study of hippocampal function. Following a reduction in NMDAR-mediated synaptic activity genes in those pathways were no longer differentially expressed. For that reason, I would expect that lateralized synaptic plasticity would no longer be observed following CPP injections between P6 and P9.

In the future, I would seek to determine whether lateralized differences in long-term synaptic plasticity are observed during early postnatal development in the rat

hippocampal formation. To test that hypothesis, I would determine whether postsynaptic responses following high frequency or theta burst stimulation of afferent input would be larger in the right hippocampal formation at P6 and in the left hippocampal formation at P9 in adult rats. More specifically, I would compare the left and right excitatory postsynaptic potentials recorded in the dentate gyrus following stimulation of the perforant path, the potentials recorded in CA3 following stimulation of mossy fiber axons or commissural axons, and in CA1 following stimulation of Schaffer collateral axons. I expect that the LTP threshold would be lower and the amount of LTP would be greater in the right hippocampal formation at P6 and in the left at P9 and in the adult rat.

Slice recordings have been used to examine differences in synaptic activity between the left and right hippocampal formation in adult rats. Using paired pulses with varying interstimulus intervals, I will be able to determine whether the lateralized expression of synaptic vesicle trafficking genes that was previously observed is likely to affect paired pulse facilitation (PPF) or depression (PPD) differently in the left and right hippocampal formation. If the lateralized expression of synaptic vesicle trafficking genes in the right hippocampal formation is functionally significant, then I would expect that short term synaptic plasticity would also be affected.

For example, at P6, synaptic vesicle trafficking genes are more highly expressed in the right hippocampal formation; therefore, I would expect that following equivalent stimulation of afferent input in either the left or right hippocampal formation (as determined by producing a presynaptic fiber volley of the same size in both sets of recordings) the slope of the second of paired field excitatory postsynaptic potentials (EPSP) would be greater in the right hippocampal formation than in left hippocampal

formation. This would likely be due to an increase in the facilitated release of neurotransmitter. Furthermore, in instances where the interstimulus interval is shorter, and the cell must rely on the reserve pool of vesicles, I would expect that at P6 the right hippocampal formation would have greater fEPSP slope as compared to the left hippocampal formation that may have a smaller reserve pool of vesicles. In contrast to P6, during normal development at P9 I would expect the opposite results. Since vesicle trafficking genes shifted to be more highly expressed in the left hippocampal formation at P9, I would expect a corresponding increase in EPSP slopes for equivalent stimulation and that PPF would be more likely to be observed in the left hippocampal formation at P9. Furthermore, since our lab previously showed that this pattern of lateralized gene expression is maintained in the adult rat, I would also expect to see similar results in the adult rat as compared to normal development at P9.

Additionally, both saline and CPP injections between P6 and P9 resulted in a loss of lateralized expression of synaptic vesicle trafficking genes at P9. Those findings suggest that following either injection procedure PPF and PPD would no longer be lateralized at P9. This finding would indicate that the injection procedure would be sufficient to eliminate the differential expression of synaptic vesicle trafficking genes and would eliminate any lateralized effect of paired-pulses that might normally be seen at P9. It would be interesting to determine whether the effect of either saline or CPP injections between P6 and P9 would be maintained in the adult.

Alternatively, the increased expression of vesicle trafficking genes could indicate that the depletion of the readily releasable and reserve pools of vesicles occurs at a faster rate following stimulation. This would result in reduced vesicular release of neurotransmitter

following repeated stimulation and might increase the likelihood of observing PPD. In that instance, I would expect that PPD would be greater in the right hippocampal formation at P6, in the left hippocampal formation during normal development at P9, and that it would remain higher in the left hippocampal formation in the adult.

I have suggested that LTP and LTD may be lateralized during rat hippocampal development. I would further specifically examine NMDAR-dependent LTP and LTD. NMDAR-dependent potentiation requires the simultaneous removal of a magnesium block and the binding of glutamate to allow entry of calcium into the postsynaptic cell. This potential for lateralized calcium influx could also account for the lateralized expression of genes corresponding to proteins in the calcium signaling pathway. To investigate that possibility, I would utilize tetanized inputs to Schaffer collateral axons in hippocampal slices to conclusively determine whether NMDAR-dependent LTP or LTD induction and maintenance are greater in the right CA1 subregion at P6 and the left CA1 subregion at P9. I would expect, given the lateralized gene expression patterns, that LTP and LTD induction and maintenance would still be greater in the left CA1 at P9 following saline injections between P6 and P9, and would not be lateralized at P9 following a reduction in NMDAR-mediated synaptic activity between P6 and P9.

It is also important to consider the lateralized expression of other receptor genes aside from the lateralized effect of a reduction in NMDAR-mediated synaptic activity that was investigated in the present study. Others have argued GABA and AMPA receptors are also extremely important in the development of the neonatal hippocampus - silent synapses are first activated by excitatory $GABA_A$ receptors and then later by AMPA receptors as the hippocampal formation begins to develop more adult-like forms of

synaptic plasticity. The $GABA_A$ receptor is more highly expressed in the right hippocampus during normal development at P9 when $GABA_A$ receptor activity shifts to become inhibitory as a result of an increase in the extracellular concentration of chloride. The specific shift in $GABA_A$ receptor gene expression suggests bicuculline, a $GABA_A$ receptor antagonist, would exhibit significant differential effects between P6 and P9 on lateralized development of the hippocampal formation. I would expect that a reduction in $GABA_A$ receptor activity would delay the development of mature hippocampal synapses. Additionally, GluR2 containing AMPARs were more highly expressed in the left hippocampal formation during normal development at P9. During early postnatal development the majority of hippocampal synapses are silent and the upregulation of AMPA receptors leads to a decrease in the number of silent synapses. The use of the AMPA receptor antagonist CNQX between P6 and P9 would block synaptic activity and prevent the decrease in silent synapses. A comparison of NMDA, GABA, and AMPA receptor-mediated synaptic activity on the maturation of hippocampal synapses and synaptic plasticity in the left and right hippocampal formation could shed light on how these neurotransmitters contribute to lateralized hippocampal development.

Possible Lateralized Activation of Signaling Pathways that Promote Development and Maturation of the Rat Hippocampal Formation

I discussed the potential for lateralized synaptic activity in the preceding section that, importantly, could lead to differential kinase and phosphorylation activity, protein synthesis, and gene expression. My findings support this possibility as genes that were shown to be differentially expressed are components of pathways known to be involved

in hippocampal growth and development: I observed lateralized expression of genes in the VEGF, Wnt, and MAPK signaling pathways.

Genes corresponding to proteins in the VEGF, Wnt, and MAPK signaling pathways were more highly expressed in the right hippocampal formation at P6. By P9, genes within the VEGF and MAPK signaling pathways were no longer differentially expressed and one gene within the Wnt signaling pathway was more highly expressed in the left hippocampal formation. In contrast, following a reduction in NMDAR-mediated synaptic activity, genes within those pathways were more highly expressed in the right hippocampal formation. Those expression patterns further support my hypothesis that the right hippocampal formation develops and matures before the left.

VEGF and Wnt signaling are known to promote neurogenesis, cell survival, and maturation. Wnt has also been shown to be involved in axis patterning and the development of the hippocampal circuit. The differential expression of genes within those pathways further supports the argument that cell growth and maturation are greater in the right hippocampal formation at P6 and the left at P9. The left-shift in gene expression that is no longer observed following a reduction in NMDAR-mediated synaptic activity suggests that a left-shift in rat hippocampal development might also be prevented.

In a previous study, NMDA, the cannonical NMDAR agonist, was shown not to cause cell death in immature hippocampal neurons during embryonic and early postnatal development, but did cause cell death in more mature neurons. This was suggested to be due to differences in the activation of the MAPK signaling pathway during hippocampal development. At P6, in immature neurons in the right hippocampal formation NMDAR activation preferentially induced the ERK component of the pathway. In contrast,

NMDAR activation at P9 in more mature neurons in the right hippocampal formation led to activation of the p38 (MAPK14) pathway that results in neurotoxicity. These results are intriguing when considered with my findings that suggest the left hippocampal formation matures later than the right. This could indicate that the shift from the preferential activation of ERK to MAPK14 signaling would occur in the right hippocampal formation before the left. If at P9, cells within the left hippocampal formation are still immature, then they might be more resistant to the neurotoxic effects of the p38 MAPK signaling pathway. For this reason, I would expect that the activation of the NMDA receptor at P9 would be more likely to induce neurotoxic effects in the right hippocampal formation when compared to the left.

Interestingly, the developmental period between P6 to P9 coincides with a switch in the predominant expression of NR2B to NR2A in NMDARs. The potential for increased maturation in the right hippocampal formation during early postnatal development could indicate that the shift in the predominant expression of NR2B to NR2A might occur in the right hippocampal formation first. If this were the case, it would suggest that those more mature neurons with a greater proportion of NR2A subunits would be differentially affected by a reduction in NMDAR-mediated synaptic activity, which in turn would preferentially trigger the lateralized activation of the p38 MAPK signaling pathway, rather than the ERK MAPK signaling pathway. My findings did not indicate that the NR2A or NR2B subunit expression was lateralized in the rat hippocampal formation. However, others have shown that the lateralized expression of the NR2B receptor in the adult rat is specific to particular layers within CA1 and is not lateralized across the entire hippocampal formation. Therefore, it would also be interesting to examine the possibility

that the NR2 receptor subunits are differentially expressed in early hippocampal development in particular subregions or layers of the hippocampal formation. To test these hypotheses, I would use specific antibodies to determine the density of NR2A and NR2B subunits in the left and right hippocampal formation in each subregion and the layers within those subregions.

Possible Effects of Lateralized Development on Later Hippocampal Function

Any delayed morphological and physiological development of the left hippocampal formation as discussed above would have important implications for the formation of hippocampal circuitry and later function of the hippocampal formation in learning and memory. It has been argued that structures within the brain that develop more slowly show greater functional plasticity. This is potentially significant when considering previous studies demonstrating that unilateral inactivation of the left hippocampus in adult and aged rats produces greater performance deficits in hippocampal-dependent learning tasks when compared to right hippocampal activation.

The delayed development of the left hippocampal formation might contribute to the greater plasticity that would support the preferential role of the left hemisphere in hippocampal-dependent learning and memory tasks. NMDAR activity has been shown to influence synapse formation during development and adult neurogenesis in the rat dentate gyrus. Therefore, a reduction in NMDAR activity that potentially delays or prevents a left-shift in hippocampal development might also affect adult hippocampal-dependent learning and memory. To assess this possibility I would determine whether CPP injections between P6 and P9 affect adult rat performance in the MWMT following either

left or right unilateral inactivation of the hippocampal formation. I expect that following saline injections between P6 and P9 unilateral inactivation of the left hippocampal formation would produce greater deficits in performance in the MWMT when compared to inactivation of the right hippocampal formation. I also expect that a reduction in NMDAR-mediated synaptic activity would produce the same deficit in performance in the MWMT regardless of which hemisphere is inactivated.

The lateralized development of the rat hippocampal formation would also have important implication to the development of neuropsychiatric disorders, including, for example, schizophrenia. Adult schizophrenics have been shown to display differences in anatomical hippocampal laterality when compared to normal individuals. A monozygotic twin study where only one of the twins was affected showed that the schizophrenic twin had greater enlargement of the lateral and third ventricles resulting in decreased cerebral volume. Decreased cerebral volume with schizophrenia is most often observed in the temporal lobe, especially the limbic system, which includes the hippocampal formation. Furthermore, the reduction in hippocampal volume and surrounding areas of the temporal lobe has been shown to be greater in the left hemisphere of schizophrenics. Lateralized hippocampal volume is not unique to schizophrenia, but changes in the lateralized development of the region may account for some of the behavioral differences that are observed with the disorder. For example, schizophrenics perform poorly on hippocampal dependent learning and memory tasks.

If one considers the studies of hippocampal asymmetry in schizophrenics and previous findings where a left-shift in cerebral activity is observed during human development, they suggest that the preferential reduction in left hippocampal volume seen with

schizophrenia may be due to a loss in the left-shift in hippocampal development. This is intriguing as it suggests that further study of the lateralized development of the human hippocampal formation is warranted if a greater understanding of the etiology of schizophrenia is desired.

A reduction in SNAP-25, a synaptic vesicle trafficking gene, was observed within the hippocampal formation of schizophrenic patients. Our lab previously showed that vesicle trafficking genes were more highly expressed in the left hippocampal formation of the adult rat. These studies indicate a potential role for altered vesicular release of neurotransmitter in the development of schizophrenia and the need for examining the lateralized expression of vesicle trafficking genes in schizophrenics to determine whether the reduction in vesicle trafficking gene expression is greater in the left hemisphere.

Interestingly, use of PCP, a drug that acts to block the NMDAR, can also mimic the behavior associated with positive symptoms observed in schizophrenia. Schizophrenics also have lower densities of the NMDA, AMPA, and Kainate glutamate receptors in the hippocampus. This could have an effect on the induction of LTP, a critical cellular process that may underlie learning and memory, in the hippocampal formation that has been shown to be dependent upon AMPA and NMDA receptor density, and could account for working memory deficits that are observed in schizophrenia. Loss of glutamate receptor density increases over the lifespan of schizophrenics indicating a potential role for glutamate receptors in the development and progression of the disease.

Activity at the N-methyl-D-aspartate (NMDA) receptor is necessary not only for LTP induction and hippocampal dependent learning and memory, but also for proper maturation of granule cells in the hippocampal formation of the rat: blockade of the

NMDA receptor using the competitive receptor antagonist CPP between P6 and P9 significantly slows the appearance of spines and the regression of immature features on granule cell dendrites during early development. These findings indicate that a reduction in NMDA receptor activity influences cellular development. Granule neurons of the dentate gyrus are known to actively undergo neurogenesis over the entire lifespan. The reduction in NMDA receptor activity that is observed over the lifespan of schizophrenics might influence the proper development of those cells and could even alter hippocampal connectivity in the adult. It has not been determined whether neurogenesis is lateralized in adults or whether there is a preferential reduction in neurogenesis in the left hemisphere of schizophrenics that might account for the loss of volumetric asymmetry seen in schizophrenic patients.

The possibility that the development and progression of schizophrenia results in a loss of hippocampal asymmetry is of particular interest. Taken together those findings suggest that further studies focused on the development of lateralization in humans and rodents might lead to better model of schizophrenia and a greater understanding of the development of the disorder. Interestingly, those studies also indicate that a loss asymmetry contributes to dramatic changes in behavior.

Conclusions

In conclusion, my findings suggest that hippocampal development is lateralized. More specifically I would suggest that cells in the right hippocampal formation are born and mature first. As a result, those cells form synaptic connections first and likely contribute to lateralized synaptic activity. This would result in the differential activation of signaling

pathways. Importantly, these processes are not entirely fixed: early postnatal experience, including a reduction in NMDAR-mediated synaptic activity, likely delays a left-shift in hippocampal development that could have serious consequences for adult learning and memory. Thus, lateralization of neurogenesis, the initial formation of synapses, activation of signaling pathways, and functional connectivity in the developing hippocampal formation could establish an initial set point that is necessary for proper hippocampal function in the adult. Perturbations in establishing that set point could contribute to the development of neuropsychiatric disorders, including schizophrenia.

Appendix A: Gene Ontology Terms Enriched in the Right Hippocampus of Male Rats at E18 Using DAVID Analysis

The following table contains gene ontology terms (GoTerms) shown to be enriched in the right hippocampus of male rats at E18 using DAVID analysis. Forty-two GoTerms listed here within the molecular function, cellular components, and biological processes gene ontology categories were statistically significant with a p-value of less than 0.05. The gene numbers indicate the number of genes within the gene set generated using SAM analysis with a false discovery rate of less than 10% that are represented within those gene ontology categories.

Molecular Function GoTerms	**Gene Numbers**	**P-Value**
GTPase activity	5	0.0000
structural molecule activity	6	0.0004
nucleoside-triphosphatase activity	5	0.0011
pyrophosphatase activity	5	0.0013
hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	5	0.0013
hydrolase activity, acting on acid anhydrides	5	0.0013
structural constituent of cytoskeleton	3	0.0021
GTP binding	4	0.0023
guanyl ribonucleotide binding	4	0.0025
guanyl nucleotide binding	4	0.0025
protein binding	10	0.0150
binding	13	0.0470
ligand-gated channel activity	2	0.0840

Cellular Components GoTerms	**Gene Numbers**	**P-Value**
macromolecular complex	10	0.0001
intracellular non-membrane-bound organelle	8	0.0003
non-membrane-bound organelle	8	0.0003
microtubule	4	0.0005
protein complex	8	0.0007
cytoplasm	11	0.0011
cytoskeletal part	5	0.0016
microtubule cytoskeleton	4	0.0031
cytoskeleton	5	0.0059
cell soma	3	0.0060
intracellular organelle part	7	0.0170
organelle part	7	0.0170
intracellular part	12	0.0170
plasma membrane	6	0.0320

intracellular	12	0.0380

Biological Processes GoTerms	**Gene Numbers**	**P-Value**
protein polymerization	4	0.0000
microtubule-based process	4	0.0010
organelle organization and biogenesis	6	0.0016
microtubule-based movement	3	0.0047
cytoskeleton-dependent intracellular transport	3	0.0062
cytoskeleton organization and biogenesis	4	0.0099
dopamine metabolic process	2	0.0190
cellular component organization and biogenesis	7	0.0200
catecholamine metabolic process	2	0.0280
phenol metabolic process	2	0.0290
neurotransmitter metabolic process	2	0.0390
cellular metabolic process	11	0.0400
primary metabolic process	11	0.0420
alcohol metabolic process	3	0.0420

Appendix B: Gene Ontology Terms Enriched in the Right Hippocampus of Male Rats at E18 Using GoMiner Analysis

The following table contains gene ontology terms (GoTerms) shown to be enriched in the right hippocampus of male rats at E18 using GoMiner analysis. 155 GoTerms listed here were statistically significant with a p-value of less than 0.05 using the two-sided Fisher's exact test.

GoTerm	P-Value
GTP binding	0.0000
GTPase activity	0.0000
guanyl nucleotide binding	0.0000
guanyl ribonucleotide binding	0.0000
intracellular non-membrane-bounded organelle	0.0000
Microtubule	0.0000
non-membrane-bounded organelle	0.0000
nucleoside-triphosphatase activity	0.0000
protein polymerization	0.0000
structural constituent of cytoskeleton	0.0000
structural molecule activity	0.0000
hydrolase activity, acting on acid anhydrides	0.0001
hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	0.0001
pyrophosphatase activity	0.0001
cytoskeleton-dependent intracellular transport	0.0002
macromolecular complex	0.0002
microtubule cytoskeleton	0.0002
microtubule-based movement	0.0002
microtubule-based process	0.0007
cytoskeletal part	0.0015
organelle organization and biogenesis	0.0015
structural constituent of ribosome	0.0020
spindle organization and biogenesis	0.0030
Ribosome	0.0042
protein complex	0.0047
Cytoskeleton	0.0049
nuclear chromosome	0.0055
cytoskeleton organization and biogenesis	0.0072
cellular protein metabolic process	0.0108
intracellular part	0.0111
Intracellular	0.0128
ribonucleoprotein complex	0.0140
adenylate cyclase binding	0.0150
Barr body	0.0150
cell tip growth	0.0150
cytoplasmic microtubule	0.0150
cytosolic large ribosomal subunit	0.0150
fat-soluble vitamin biosynthetic process	0.0150

gamma-tubulin complex	0.0150
GMP salvage	0.0150
homogentisate phytyltransferase activity	0.0150
homogentisate prenyltransferase activity	0.0150
hypoxanthine phosphoribosyltransferase activity	0.0150
IMP salvage	0.0150
large ribosomal subunit	0.0150
meiotic spindle organization and biogenesis	0.0150
metabolic compound salvage	0.0150
MHC class I protein binding	0.0150
MHC protein binding	0.0150
microtubule nucleation	0.0150
microtubule organizing center part	0.0150
negative regulation of interleukin-1 beta production	0.0150
negative regulation of interleukin-1 production	0.0150
nucleoside metabolic process	0.0150
nucleoside salvage	0.0150
nucleotide salvage	0.0150
pericentriolar material	0.0150
phloem loading	0.0150
phragmoplast formation	0.0150
polar microtubule	0.0150
positive regulation of heart rate in baroreceptor response to decreased systemic arterial blood pressure	0.0150
purine nucleoside metabolic process	0.0150
purine nucleotide salvage	0.0150
purine ribonucleoside metabolic process	0.0150
purine ribonucleoside salvage	0.0150
purine salvage	0.0150
ribonucleoside metabolic process	0.0150
root epidermal cell differentiation	0.0150
root hair cell differentiation	0.0150
root hair cell tip growth	0.0150
root hair elongation	0.0150
sensory perception of sour taste	0.0150
sodium channel inhibitor activity	0.0150
sodium channel regulator activity	0.0150
spindle microtubule	0.0150
spindle pole body	0.0150
stomatal complex development	0.0150
stomatal complex morphogenesis	0.0150
toxin binding	0.0150
trichoblast differentiation	0.0150
trichoblast maturation	0.0150
virion binding	0.0150
vitamin E biosynthetic process	0.0150

vitamin E metabolic process	0.0150
X chromosome	0.0150
cellular macromolecule metabolic process	0.0156
hydrolase activity	0.0159
protein metabolic process	0.0189
intracellular organelle part	0.0205
organelle part	0.0227
Chromosome	0.0276
M phase of mitotic cell cycle	0.0276
Mitosis	0.0276
antigen binding	0.0297
antigen processing and presentation of peptide antigen via MHC class I	0.0297
asexual reproduction	0.0297
baroreceptor response to decreased systemic arterial blood pressure	0.0297
chloride channel regulator activity	0.0297
cis-trans isomerase activity	0.0297
cytokinesis by cell plate formation	0.0297
developmental cell growth	0.0297
embryo sac development	0.0297
formation of actomyosin apparatus involved in cytokinesis	0.0297
fruiting body development	0.0297
fruiting body development in response to starvation	0.0297
gametophyte development	0.0297
generation of ovulation cycle rhythm	0.0297
interleukin-1 beta production	0.0297
negative regulation of tumor necrosis factor production	0.0297
nuclear heterochromatin	0.0297
Nucleosome	0.0297
peptidyl-prolyl cis-trans isomerase activity	0.0297
prenyltransferase activity	0.0297
regulation of interleukin-1 beta production	0.0297
regulation of tumor necrosis factor production	0.0297
sex chromatin	0.0297
sex chromosome	0.0297
sorocarp development	0.0297
spindle assembly	0.0297
spindle pole	0.0297
tRNA binding	0.0297
tubulin complex	0.0297
vitamin biosynthetic process	0.0297
cellular biosynthetic process	0.0307
intracellular organelle	0.0333
Organelle	0.0346
M phase	0.0370

microtubule cytoskeleton organization and biogenesis	0.0370
cellular localization	0.0379
protein kinase binding	0.0403
Translation	0.0438
antigen processing and presentation of peptide antigen	0.0443
behavioral response to ethanol	0.0443
Cognition	0.0443
cytokinetic process	0.0443
cytosolic ribosome	0.0443
epidermal cell differentiation	0.0443
interleukin-1 production	0.0443
leaf development	0.0443
MHC class I protein complex	0.0443
MHC protein complex	0.0443
mitotic spindle organization and biogenesis	0.0443
negative regulation of interleukin-6 production	0.0443
norepinephrine secretion	0.0443
nuclear chromatin	0.0443
positive regulation of angiogenesis	0.0443
purine nucleotide biosynthetic process	0.0443
regulation of interleukin-1 production	0.0443
regulation of norepinephrine secretion	0.0443
regulation of synaptic transmission, dopaminergic	0.0443
ribosomal subunit	0.0443
ribosome biogenesis and assembly	0.0443
root morphogenesis	0.0443
tumor necrosis factor production	0.0443
unidimensional cell growth	0.0443

Appendix C: Gene Ontology Terms Enriched in the Left Hippocampal Formation of Male Rats at P9 Following Saline Injections Between P6 and P9.

The following table contains gene ontology terms (GoTerms) shown to be enriched in the left hippocampus of male rats at P9 following saline injections between P6 and P9 using DAVID analysis. 113 GoTerms listed here within the molecular function, cellular components, and biological processes gene ontology categories were statistically significant with a p-value of less than 0.05. The gene numbers indicate the number of genes within the gene set generated using SAM analysis with a false discovery rate of less than 10% that are represented within those gene ontology categories.

Molecular Function GoTerms	Gene Numbers	P-Value
protein binding	30	0.0000
auxiliary transport protein activity	5	0.0000
binding	37	0.0011
structural constituent of cytoskeleton	4	0.0013
protein dimerization activity	6	0.0032
protein heterodimerization activity	4	0.0130
cation transmembrane transporter activity	6	0.0170
transcription activator activity	4	0.0450
neurotransmitter binding	3	0.0450
ion transmembrane transporter activity	6	0.0460

Cellular Components GoTerms	Gene Numbers	P-Value
plasma membrane	18	0.0000
intrinsic to plasma membrane	12	0.0001
integral to plasma membrane	11	0.0003
neuron projection	6	0.0006
synapse	6	0.0006
plasma membrane part	13	0.0008
soluble fraction	6	0.0011
axon	4	0.0028
cell projection part	4	0.0036
cytoskeletal part	7	0.0041
dendrite	4	0.0056
cytoskeleton	8	0.0057
cell projection	6	0.0074
cell fraction	9	0.0077
extracellular space	12	0.0085
extracellular region part	12	0.0130
cell surface	4	0.0160
extracellular region	12	0.0290
anchored to plasma membrane	2	0.0440
cell soma	3	0.0450
cytoplasm	18	0.0490

Biological Process GoTerms	Gene Numbers	P-Value
transmission of nerve impulse	13	0.0000
synaptic transmission	12	0.0000
cell-cell signaling	14	0.0000
nervous system development	14	0.0000
regulation of neurotransmitter levels	7	0.0000
dopamine metabolic process	4	0.0000
catecholamine metabolic process	4	0.0001
phenol metabolic process	4	0.0001
system development	16	0.0001
biological regulation	25	0.0001
neurogenesis	8	0.0002
anatomical structure development	17	0.0002
regulation of biological quality	11	0.0002
multicellular organismal development	17	0.0002
neurotransmitter metabolic process	4	0.0002
chemical homeostasis	7	0.0003
neuron differentiation	7	0.0003
homeostatic process	8	0.0004
generation of neurons	7	0.0007
cell development	12	0.0008
cellular ion homeostasis	6	0.0009
cellular chemical homeostasis	6	0.0009
developmental process	19	0.0009
multicellular organismal process	24	0.0011
ion homeostasis	6	0.0013
biogenic amine metabolic process	4	0.0015
neurological system process	15	0.0020
cell communication	24	0.0021
system process	16	0.0022
localization	18	0.0024
G-protein signaling, coupled to IP3 second messenger (phospholipase C activating)	4	0.0027
cellular homeostasis	6	0.0030
alcohol metabolic process	6	0.0034
regulation of biological process	20	0.0044
phosphoinositide-mediated signaling	4	0.0045
amino acid derivative metabolic process	4	0.0050
positive regulation of cellular metabolic process	6	0.0058
central nervous system development	5	0.0064
positive regulation of metabolic process	6	0.0077
cell differentiation	12	0.0078
cellular developmental process	12	0.0078
positive regulation of cellular process	9	0.0085
cellular calcium ion homeostasis	4	0.0089
calcium ion homeostasis	4	0.0089
cellular component organization and biogenesis	15	0.0091
aromatic compound metabolic process	4	0.0091

synapse organization and biogenesis	3	0.0110
brain development	4	0.0110
cellular metal ion homeostasis	4	0.0110
metal ion homeostasis	4	0.0110
protein polymerization	3	0.0130
organ development	10	0.0140
anatomical structure morphogenesis	9	0.0140
regulation of a molecular function	6	0.0140
positive regulation of biological process	9	0.0150
dopamine biosynthetic process	2	0.0160
di-, tri-valent inorganic cation homeostasis	4	0.0180
cellular di-, tri-valent inorganic cation homeostasis	4	0.0180
behavior	5	0.0190
cellular morphogenesis during differentiation	4	0.0210
transport	14	0.0220
cellular process	40	0.0220
tissue development	5	0.0250
elevation of cytosolic calcium ion concentration	3	0.0260
cation homeostasis	4	0.0260
cellular cation homeostasis	4	0.0260
amino acid and derivative metabolic process	5	0.0270
cytosolic calcium ion homeostasis	3	0.0280
cell fate commitment	3	0.0280
developmental growth	3	0.0290
establishment of localization	14	0.0290
neurotransmitter secretion	3	0.0310
neuron development	4	0.0330
catecholamine biosynthetic process	2	0.0340
positive regulation of catalytic activity	4	0.0370
extracellular structure organization and biogenesis	3	0.0380
positive regulation of transcription, DNA-dependent	4	0.0390
regulation of catalytic activity	5	0.0390
amine metabolic process	5	0.0400
regulated secretory pathway	3	0.0400
regulation of transcription from RNA polymerase II promoter	5	0.0430
nitrogen compound metabolic process	5	0.0480

Appendix D: Gene Ontology Terms Enriched in the Right Hippocampal Formation of Male Rats at P9 Following Saline Injections Between P6 and P9.

The following table contains gene ontology terms (GoTerms) shown to be enriched in the right hippocampus of male rats at P9 following saline injections between P6 and P9 using DAVID analysis. 90 GoTerms listed here within the molecular function, cellular components, and biological processes gene ontology categories were statistically significant with a p-value of less than 0.05. The gene numbers indicate the number of genes within the gene set generated using SAM analysis with a false discovery rate of less than 10% that are represented within those gene ontology categories.

Molecular Function GoTerms	Gene Numbers	P-Value
protein binding	16	0.0021
double-stranded DNA binding	3	0.0072
metal ion transmembrane transporter activity	4	0.0110
iron ion transmembrane transporter activity	2	0.0120
binding	21	0.0130
structure-specific DNA binding	3	0.0140
ion transmembrane transporter activity	5	0.0210
DNA binding	7	0.0250
protein heterodimerization activity	3	0.0300
transcription regulator activity	6	0.0350
substrate-specific transmembrane transporter activity	5	0.0360
transcription factor activity	5	0.0370
transition metal ion transmembrane transporter activity	2	0.0430
specific RNA polymerase II transcription factor activity	2	0.0430
transporter activity	6	0.0440
cation transmembrane transporter activity	4	0.0490
transmembrane transporter activity	5	0.0490

Cellular Components GoTerms	Gene Numbers	P-Value
protein complex	10	0.0010
macromolecular complex	11	0.0014
intracellular part	18	0.0068
cytoskeleton	6	0.0069
nucleus	11	0.0100
intracellular organelle	16	0.0110
organelle	16	0.0120
intracellular	18	0.0210
intracellular membrane-bound organelle	14	0.0210
membrane-bound organelle	14	0.0210
cytoplasm	12	0.0400

Biological Processes GoTerms	Gene Numbers	P-Value
regulation of transcription from RNA polymerase II promoter	7	0.0001
transcription from RNA polymerase II promoter	7	0.0003
cellular ion homeostasis	5	0.0007
cellular chemical homeostasis	5	0.0007
homeostatic process	6	0.0008
positive regulation of cellular process	8	0.0008
ion homeostasis	5	0.0010
cellular iron ion homeostasis	3	0.0013
iron ion homeostasis	3	0.0013
positive regulation of biological process	8	0.0014
chemical homeostasis	5	0.0016
cellular homeostasis	5	0.0019
regulation of biological quality	7	0.0026
di-, tri-valent inorganic cation homeostasis	4	0.0034
cellular di-, tri-valent inorganic cation homeostasis	4	0.0034
positive regulation of transcription from RNA polymerase II promoter	4	0.0034
cell differentiation	9	0.0035
cellular developmental process	9	0.0035
regulation of cell differentiation	4	0.0037
macromolecule metabolic process	17	0.0039
metal ion transport	5	0.0046
cation homeostasis	4	0.0050
cellular cation homeostasis	4	0.0050
biological regulation	14	0.0055
system development	9	0.0056
primary metabolic process	18	0.0073
positive regulation of transcription, DNA-dependent	4	0.0078
positive regulation of cell proliferation	4	0.0080
cation transport	5	0.0098
developmental process	11	0.0110
regulation of apoptosis	5	0.0110
regulation of programmed cell death	5	0.0120
regulation of developmental process	4	0.0120
cell development	7	0.0130
positive regulation of transcription	4	0.0130
anatomical structure development	9	0.0140
positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	4	0.0150
multicellular organismal development	9	0.0170
regulation of biological process	12	0.0180

regulation of gene expression	8	0.0180
cellular metabolic process	17	0.0210
positive regulation of cellular metabolic process	4	0.0250
regulation of cellular metabolic process	8	0.0250
apoptosis	5	0.0270
regulation of transcription, DNA-dependent	7	0.0280
metabolic process	18	0.0280
programmed cell death	5	0.0280
response to stress	6	0.0290
positive regulation of metabolic process	4	0.0300
di-, tri-valent inorganic cation transport	3	0.0300
RNA metabolic process	8	0.0300
cell death	5	0.0320
death	5	0.0320
regulation of metabolic process	8	0.0320
ion transport	5	0.0340
cell proliferation	5	0.0350
transcription, DNA-dependent	7	0.0360
RNA biosynthetic process	7	0.0360
nervous system development	5	0.0400
regulation of transcription	7	0.0420
gene expression	9	0.0460
regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	7	0.0480

Appendix E: Gene Ontology Terms Enriched in the Right Hippocampal Formation of Male Rats at P9 Following CPP Injections Between P6 and P9.

The following table contains gene ontology terms (GoTerms) shown to be enriched in the right hippocampal formation of male rats at P9 following a reduction in NMDAR activity using CPP injections between P6 and P9. Using DAVID analysis, 8 GoTerms listed here within the molecular function, cellular components, and biological processes gene ontology categories were statistically significant with a p-value of less than 0.05. The gene numbers indicate the number of genes within the gene set generated using SAM analysis with a false discovery rate of less than 10% that are represented within those gene ontology categories.

Molecular Function GoTerms	Gene Numbers	P-Value
GABA receptor activity	3	0.0001
GABA-B receptor activity	2	0.0068
metabotropic glutamate, GABA-B-like receptor activity	2	0.0270
glutamate receptor activity	2	0.0320

Cellular Components GoTerms	Gene Numbers	P-Value
postsynaptic membrane	3	0.0004
synapse part	3	0.0006
synapse	3	0.0020

Biological Processes GoTerms	Gene Numbers	P-Value
gamma-aminobutyric acid signaling pathway	2	0.0063

Appendix F: Gene Ontology Terms Enriched in the Right Hippocampal Formation of Male Rats at P6

The following table contains gene ontology terms (GoTerms) shown to be enriched in the right hippocampus of male rats at P6 during normal development using DAVID analysis. 155 GoTerms listed here within the molecular function, cellular components, and biological processes gene ontology categories were statistically significant with a p-value of less than 0.05. The gene numbers indicate the number of genes within the gene set generated using SAM analysis with a false discovery rate of less than 10% that are represented within those gene ontology categories.

Biological Process GoTerms	Gene Numbers	P-Value
regulation of multicellular organismal process	13	0.000
anatomical structure development	18	0.000
regulation of biological quality	14	0.000
system development	17	0.000
developmental process	19	0.000
cell differentiation	14	0.000
cellular developmental process	14	0.000
generation of neurons	9	0.000
neuron differentiation	8	0.000
neurogenesis	9	0.000
multicellular organismal development	17	0.000
cell development	9	0.001
response to organic substance	10	0.001
regulation of cell communication	10	0.001
regulation of growth	6	0.002
biological regulation	26	0.002
regulation of system process	6	0.002
neuron development	6	0.003
positive regulation of axonogenesis	3	0.004
response to stress	11	0.005
transport	14	0.005
establishment of localization	14	0.006
nervous system development	9	0.007
cellular component organization	13	0.007
response to inorganic substance	5	0.008
response to external stimulus	8	0.008
response to nutrient levels	5	0.009
regulation of transport	6	0.010
striated muscle cell development	3	0.011
response to extracellular stimulus	5	0.011
positive regulation of axon regeneration	2	0.012
positive regulation of neuron projection regeneration	2	0.012
regulation of biological process	23	0.012
ion transport	7	0.012
regulation of synaptic transmission	4	0.012
positive regulation of developmental process	5	0.012
muscle cell development	3	0.013

positive regulation of cell projection organization	3	0.014
regulation of transmission of nerve impulse	4	0.014
cellular process	30	0.015
response to stimulus	19	0.016
organ development	11	0.016
regulation of cellular catabolic process	3	0.017
regulation of axonogenesis	3	0.017
regulation of neurological system process	4	0.017
regulation of axon regeneration	2	0.020
regulation of neuron projection regeneration	2	0.020
localization	14	0.021
feeding behavior	3	0.021
positive regulation of neurogenesis	3	0.022
cellular component morphogenesis	5	0.023
multicellular organismal process	20	0.024
positive regulation of neuron projection development	2	0.026
positive regulation of cell development	3	0.027
positive regulation of growth	3	0.028
regulation of cellular component organization	5	0.028
striated muscle cell differentiation	3	0.028
metal ion transport	5	0.029
positive regulation of transport	4	0.030
regulation of neuron projection development	3	0.030
regulation of anatomical structure morphogenesis	4	0.031
response to chemical stimulus	14	0.031
regulation of cell morphogenesis involved in differentiation	3	0.031
regulation of cellular response to stress	3	0.031
desensitization of G-protein coupled receptor protein signaling pathway	2	0.032
adaptation of signaling pathway	2	0.032
regulation of cellular protein metabolic process	5	0.032
protein amino acid phosphorylation	6	0.033
tissue development	6	0.034
regulation of localization	6	0.034
behavior	5	0.034
regulation of secretion	4	0.034
regulation of catabolic process	3	0.034
phosphate metabolic process	7	0.035
phosphorus metabolic process	7	0.035
positive regulation of multicellular organismal process	4	0.036
positive regulation of cell differentiation	4	0.037
positive regulation of axon extension	2	0.037
vesicle-mediated transport	5	0.038
regulation of response to stimulus	5	0.039
glutamate metabolic process	2	0.040
regulation of cytoskeleton organization	3	0.040
regulation of cell projection organization	3	0.043

glutamine family amino acid biosynthetic process	2	0.043
regulation of systemic arterial blood pressure by renin-angiotensin	2	0.043
neuron projection development	4	0.045
peptidyl-threonine phosphorylation	2	0.046
muscle cell differentiation	3	0.046
calcium ion transport	3	0.046
regulation of cellular localization	4	0.047
positive regulation of secretion	3	0.047
regulation of protein modification process	4	0.047
enzyme linked receptor protein signaling pathway	4	0.048
negative regulation of microtubule depolymerization	2	0.049
regulation of microtubule depolymerization	2	0.049

Cellular Components GoTerms	**Gene Numbers**	**P-Value**
cell fraction	14	0.000
cytoplasm	32	0.000
membrane fraction	12	0.000
insoluble fraction	12	0.000
plasma membrane	20	0.000
cytoplasmic part	27	0.000
soluble fraction	8	0.000
plasma membrane part	14	0.000
cytosol	12	0.000
neuron projection	8	0.000
axon	6	0.000
microtubule cytoskeleton	7	0.001
cytoskeletal part	9	0.001
cell soma	6	0.001
cell projection part	6	0.001
cell projection	9	0.001
internal side of plasma membrane	5	0.001
cytoplasmic vesicle	8	0.001
vesicle	8	0.002
intracellular organelle	29	0.003
extracellular region part	8	0.003
organelle	29	0.003
intracellular part	32	0.003
cytoplasmic membrane-bounded vesicle	7	0.003
membrane-bounded vesicle	7	0.004
synapse	6	0.004
cytoskeleton	9	0.004
intracellular membrane-bounded organelle	26	0.005
membrane-bounded organelle	26	0.006
microsome	5	0.006
vesicular fraction	5	0.007
centrosome	4	0.007

microtubule organizing center	4	0.010
microtubule	4	0.011
extracellular space	6	0.012
intracellular	32	0.013
growth cone	3	0.015
site of polarized growth	3	0.015
integral to plasma membrane	5	0.018
intracellular organelle part	16	0.019
organelle part	16	0.021
intrinsic to plasma membrane	5	0.022
extracellular region	9	0.024
dendrite	4	0.028
membrane	25	0.029
Golgi apparatus	6	0.038
organelle membrane	7	0.046

Molecular Function GoTerms	**Gene Numbers**	**P-Value**
protein binding	35	0.000
protein kinase binding	6	0.000
kinase binding	6	0.000
binding	37	0.001
protein domain specific binding	6	0.002
enzyme binding	6	0.009
receptor binding	7	0.013
protein N-terminus binding	3	0.020
glutamate binding	2	0.029
calcium ion binding	6	0.031
calmodulin binding	3	0.031
substrate-specific transmembrane transporter activity	6	0.043
calcium channel regulator activity	2	0.044

Appendix G: Gene Ontology Terms Enriched in the Left Hippocampal Formation of Male Rats at P6

The following table contains gene ontology terms (GoTerms) shown to be enriched in the left hippocampus of male rats at P9 during normal development. Using DAVID analysis, 125 GoTerms listed here within the molecular function, cellular components, and biological processes gene ontology categories were statistically significant with a p-value of less than 0.05. The gene numbers indicate the number of genes within the gene set generated using SAM analysis with a false discovery rate of less than 1% that are represented within those gene ontology categories.

Biological Process GoTerms	Gene Numbers	P-Value
glycolysis	5	0.000
glucose catabolic process	5	0.000
hexose catabolic process	5	0.000
monosaccharide catabolic process	5	0.000
cellular carbohydrate catabolic process	5	0.000
alcohol catabolic process	5	0.000
protein polymerization	4	0.000
carbohydrate catabolic process	5	0.000
generation of precursor metabolites and energy	6	0.000
carbohydrate phosphorylation	3	0.000
glucose metabolic process	5	0.000
hexose metabolic process	5	0.000
alcohol metabolic process	6	0.000
monosaccharide metabolic process	5	0.000
spindle organization	3	0.001
cellular protein complex assembly	4	0.001
cellular process	19	0.001
cellular component organization	10	0.001
cellular carbohydrate metabolic process	5	0.002
neurogenesis	6	0.002
microtubule-based process	4	0.003
protein complex biogenesis	5	0.003
protein complex assembly	5	0.003
behavior	5	0.003
memory	3	0.003
cell morphogenesis involved in neuron differentiation	4	0.003
neuron differentiation	5	0.004
carbohydrate metabolic process	5	0.005
cellular macromolecular complex assembly	4	0.005
cell morphogenesis involved in differentiation	4	0.005
cellular component biogenesis	6	0.005
cellular macromolecular complex subunit organization	4	0.007
positive regulation of glycolysis	2	0.008
macromolecular complex assembly	5	0.008
microtubule-based movement	3	0.008
catabolic process	6	0.008

regulation of ion transport	3	0.009
macromolecular complex subunit organization	5	0.010
microtubule cytoskeleton organization	3	0.010
generation of neurons	5	0.011
nervous system development	6	0.013
cell morphogenesis	4	0.013
neuron development	4	0.014
regulation of glycolysis	2	0.015
cell development	5	0.016
spindle assembly	2	0.016
learning or memory	3	0.016
cellular component morphogenesis	4	0.018
cellular component assembly	5	0.020
M phase	3	0.028
regulation of cellular carbohydrate catabolic process	2	0.030
positive regulation of glucose metabolic process	2	0.030
regulation of carbohydrate catabolic process	2	0.030
regulation of transport	4	0.030
positive regulation of cellular carbohydrate metabolic process	2	0.034
positive regulation of carbohydrate metabolic process	2	0.034
regulation of ion transmembrane transporter activity	2	0.034
regulation of ion transmembrane transport	2	0.035
regulation of transmembrane transporter activity	2	0.037
positive regulation of insulin secretion	2	0.038
regulation of transmembrane transport	2	0.038
neuron projection morphogenesis	3	0.041
regulation of transporter activity	2	0.044
pigment biosynthetic process	2	0.046
positive regulation of transport	3	0.047
cell cycle phase	3	0.047
regulation of generation of precursor metabolites and energy	2	0.047
positive regulation of peptide secretion	2	0.047
cell projection morphogenesis	3	0.049

Cellular Components GoTerms	**Gene Numbers**	**P-Value**
cytosol	11	0.000
cytoplasmic part	17	0.000
cytoskeleton	9	0.000
cell fraction	9	0.000
cytoskeletal part	8	0.000
intracellular organelle part	14	0.000
intracellular non-membrane-bounded organelle	11	0.000
non-membrane-bounded organelle	11	0.000
organelle part	14	0.000
cytoplasm	18	0.000
protein complex	11	0.000
macromolecular complex	12	0.001

intracellular part	20	0.001
microtubule	4	0.002
intracellular organelle	18	0.002
organelle	18	0.002
intracellular	20	0.003
neuron projection	5	0.004
lamin filament	2	0.008
organelle envelope	5	0.009
envelope	5	0.009
nuclear lamina	2	0.009
soluble fraction	4	0.010
organelle membrane	6	0.012
microtubule cytoskeleton	4	0.018
cell projection	5	0.022
cytoplasmic microtubule	2	0.023
insoluble fraction	5	0.025
nuclear inner membrane	2	0.026
axon	3	0.036
mitochondrion	6	0.036

Molecular Function GoTerms	**Gene Numbers**	**P-Value**
hexokinase activity	4	0.000
carbohydrate kinase activity	4	0.000
glucose binding	3	0.000
GTPase activity	4	0.000
nucleotide binding	10	0.000
purine ribonucleotide binding	9	0.000
ribonucleotide binding	9	0.000
purine nucleotide binding	9	0.001
monosaccharide binding	3	0.002
glucokinase activity	2	0.003
enzyme binding	5	0.004
nucleoside-triphosphatase activity	5	0.005
pyrophosphatase activity	5	0.006
hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	5	0.006
structural molecule activity	5	0.006
hydrolase activity, acting on acid anhydrides	5	0.006
GTP binding	4	0.008
guanyl ribonucleotide binding	4	0.009
guanyl nucleotide binding	4	0.009
catalytic activity	12	0.017
protein binding	15	0.025
binding	19	0.029
sugar binding	3	0.032
structural constituent of cytoskeleton	2	0.035
transferase activity	6	0.046

Appendix H: Gene Ontology Terms Enriched in the Right Hippocampal Formation of Male Rats at P9

The following table contains gene ontology terms (GoTerms) shown to be enriched in the right hippocampus of male rats at P9 following saline injections between P6 and P9 using DAVID analysis. 16 GoTerms listed here within the molecular function and biological processes gene ontology categories were statistically significant with a p-value of less than 0.05. The gene numbers indicate the number of genes within the gene set generated using SAM analysis with a false discovery rate of less than 10% that are represented within those gene ontology categories.

Biological Process GoTerms	Gene Numbers	P-Value
positive regulation of interferon-gamma production	2	0.007
regulation of interferon-gamma production	2	0.015
cytokine production	2	0.023
chloride transport	2	0.024
adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	2	0.031
adaptive immune response	2	0.031
positive regulation of T cell activation	2	0.034
positive regulation of cytokine production	2	0.035
inorganic anion transport	2	0.038
immune system process	3	0.041
response to protein stimulus	2	0.044
positive regulation of lymphocyte activation	2	0.045
response to temperature stimulus	2	0.048
positive regulation of leukocyte activation	2	0.049

Molecular Function GoTerms	Gene Numbers	P-Value
cell surface binding	2	0.010
protein binding	6	0.028

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