Mechanisms by Which Early Nutrition Influences Spatial Memory, Adult Neurogenesis,

and Response to Hippocampal Injury

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Psychology and Neuroscience in the Graduate School of Duke University

ABSTRACT

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ABSTRACT

Altered dietary availability of the vital nutrient choline during early development leads to persistent changes in brain and behavior throughout adulthood. Prenatal choline supplementation during embryonic days (ED) 12-17 of the rodent gestation period enhances memory capacity and precision and hippocampal plasticity in adulthood, and protects against spatial learning and memory deficits shortly after excitotoxic seizures, whereas prenatal choline deficiency can compromise hippocampal memory and plasticity in adulthood. Recent evidence from our laboratory has determined that lifelong proliferation of newborn neurons in the adult hippocampus, a feature of adult hippocampal plasticity that has been implicated in some aspects of learning and memory, is modulated by early choline availability. Prenatal choline's effects on adult neurogenesis may be one mechanism for diet-induced cognitive changes throughout life and in response to injury, although little is known about the mechanisms underlying how prenatal choline alters adult neurogenesis or the neural mechanisms underlying prenatal choline supplementation's protection against cognitive deficits after seizures. To address these issues, the present set of experiments investigated how prenatal choline availability modulates specific properties of neurogenesis in the adult brain (in the intact brain and in response to injury), as well as hippocampal markers known to change in response to excitotoxin-induced seizures, and sought to relate changes in neurogenesis and in neuropathological markers following injury to changes in performance on spatial learning and memory tasks. Subjects in each experiment were adult offspring from rat dams that

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received either a control diet or diet supplemented with choline chloride or deficient of choline on ED 12-17. To measure neurogenesis, rats were given injections of the mitotic marker bromodeoxyurdine to label dividing cells in the hippocampus. Prenatal choline supplementation enhanced several properties of basal adult hippocampal neurogenesis (cell division and survival, neural stem/progenitor cell phenotype and proliferative capacity, trophic support), and this increase was associated with improvements in spatial working memory retention in a delayed-matching-to-place water maze task. In contrast, prenatal choline deficiency had little effect on basal adult hippocampal neurogenesis, and no effect on spatial memory performance. Prenatal choline supplementation also enhanced olfactory bulb neurogenesis without altering cell proliferation in the subventricular zone, while prenatal choline deficiency had no effect on either measure, showing for the first time that prenatal choline's effects on adult neurogenesis is similarly expressed in another distinct neurogenic region of the adult brain. Altered prenatal choline availability also modulated the hippocampal response to kainic acid-induced seizures where supplementation attenuated while deficiency had no effect on the injuryinduced proliferative response of the dentate gyrus shortly after injury. Prenatal choline supplementation also attenuated other markers of hippocampal neuropathology shortly after seizures and promoted the long-term hippocampal recovery from seizures months after injury, including rescuing declines in adult hippocampal neurogenesis and in spatial memory performance in a standard water maze task. Taken together, these findings demonstrate a robust neuroprotective effect of prenatal choline supplementation that may

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be driven by enhanced adult hippocampal plasticity and trophic support prior to injury, and shed light on the mechanisms underlying how prenatal choline availability alters adult hippocampal neurogenesis, which may contribute to changes in memory capacity and precision both throughout life and following neural assault.

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GENERAL INTRODUCTION

A growing body of literature from animal and human studies points to nutrient availability during early development as a key factor influencing brain development that programs neural and cognitive function in adulthood as well as vulnerability and resilience to disease throughout life (e.g., Rush, 1994; Zeisel, 2004; Benton, 2008; Corniola et al., 2008; Carlson et al., 2009; Coyle et al., 2009; White et al., 2009). The focus of these studies is the long-term effects on the gestational availability of the vital nutrient, choline, on brain and cognitive function. Altered dietary choline availability early in life leads to lasting modifications in memory capacity and precision across the lifespan, as well as persistent changes in features of adult hippocampal plasticity known to influence learning and memory function. For example, prenatal choline supplementation during embryonic days (ED) 12-17 of the rodent gestation period enhances hippocampal function (i.e., cognitive behavior and neuroplasticity) in adulthood and confers protection against age-related and injury-induced declines in spatial memory and hippocampal plasticity (Yang et al., 2000; Holmes et al., 2002; Meck and Williams, 2003; Glenn et al., 2007; Glenn et al., 2008b; Meck et al., 2008; Wong-Goodrich et al., 2008a). In contrast, prenatal choline deficiency during ED 12-17 can compromise hippocampal function and plasticity in adulthood (Meck and Williams, 1997c; Meck and Williams, 2003; Glenn et al., 2007; Wong-Goodrich et al., 2008a). These data suggest that alterations in dietary choline availability during development may produce lasting changes in brain plasticity such that the cognitive outcome in adulthood, old age, and in diseased states is altered.

The studies that comprise this dissertation focus on one specific feature of hippocampal plasticity in the adult brain, neurogenesis. The dentate gyrus of the hippocampus is one of two undisputed brain regions known to exhibit adult neurogenesis in the mammalian brain (Altman and Das, 1965), suggesting that this type of neural plasticity might be adaptive and functionally relevant for cognition. Accumulating evidence has demonstrated a role for adult hippocampal neurogenesis in aspects of spatial discrimination/pattern separation (Clelland et al., 2009) and learning and memory (e.g., Shors et al., 2002; Snyder et al., 2005; Deng et al., 2009; Kitamura et al., 2009), and has indicated that adult hippocampal neurogenesis is particularly sensitive to a number of environmental and physiological factors (for review, see Zhao et al., 2008). Recent work from our laboratory has determined that prenatal choline supplementation leads to enhanced basal levels of dentate cell/neuronal proliferation in young, middle-aged, and old rats (Glenn et al., 2007; Glenn et al., 2008b; Wong-Goodrich et al., 2008a). In contrast, adult rats that were prenatally deficient of choline are unable to upregulate neurogenesis in response to an environmentally enriching experience (Glenn et al., 2007), suggesting that specific cellular and molecular properties of adult hippocampal neurogenesis may be modified by early choline availability. Prenatally choline supplemented rats also have increased expression of neurotrophic/growth factors that promote neurogenesis (Sandstrom et al., 2002; Glenn et al., 2007; Wong-Goodrich et al., 2008a), and this enhancement in growth factor expression also continues into middle- and old age (Glenn et al., 2008b; Wong-Goodrich et al., 2008a).

These data have led to our hypothesis that changes in hippocampal neurogenesis and trophic support as a result of altered prenatal choline availability may partially underlie diet-induced alterations in performance on tasks of hippocampal learning and memory. Altered hippocampal plasticity of this sort may also be a potential mechanism for prenatal choline supplementation's protection against learning and memory deficits observed following a neural assault, like prolonged seizures (Yang et al., 2000; Holmes et al., 2002). The overarching goal of these studies was to address the issue of how prenatal choline availability modulates properties of neurogenesis in the adult brain (in the intact brain and in response to injury), which may alter the hippocampal network to produce changes in learning and memory function. This introduction provides: 1) a background on the nutrient choline, its biological functions, and its effects (prenatal availability) on cognition and hippocampal plasticity, 2) a discussion of why adult hippocampal neurogenesis may be one potential mechanism for the effects of altered prenatal choline status on adult cognition, 3) a review of the known properties of adult hippocampal neurogenesis, including stem cell properties and granule cell development, how neurogenesis reacts to intrinsic and extrinsic stimuli, and functional contributions to behavior/cognition in various contexts, and 4) a summary of the dissertation experiments.

Why Choline?

Choline (trimethyl-beta-hydroxy-ethylammonium) is a quaternary ammonium compound that is an essential nutrient for animals and humans (Zeisel and Blusztajn, 1994), and is extremely critical early in development because it is needed in large quantities to make the structural phospholipids found in all biological membranes (Blusztajn et al., 1998). Choline is also the precursor of the neurotransmitter,

acetylcholine (ACh), and is oxidized to betaine, which serves as a methyl donor that can influence the regulation of gene expression (Blusztajn and Wurtman, 1983; Blusztajn, 1998). Pregnancy and lactation place high demands on a mother's choline supply, as both the placenta and mammary glands selectively take choline from maternal plasma and transfer it to the developing infant (Zeisel and Blusztajn, 1994; Zeisel, 2004; Zeisel, 2006). Dietary intake of choline is crucial because only about 20% of one's choline supply is made directly in the body while 80% comes from the diet (Sundler and Akesson, 1975; Yao and Vance, 1988). Now over a decade ago, the Food and Nutrition Board of the Institute of Medicine-United States National Academy of Sciences issued a report on B vitamins that provided recommendations for the adequate intake of choline (Food and Nutrition Board of the Institute of Medicine, 1998). This recommendation was based, in part, on studies showing that altered choline availability during early development in rodents has considerable effects on cognitive performance throughout the lifespan (Meck et al., 1988; Meck et al., 1989; Meck and Williams, 2003; McCann et al., 2006; Meck et al., 2008).

The availability of choline during ED 12 through 17 of the 22-day rat gestation period has profound effects on cognitive performance. Prenatally choline supplemented rats show lifelong improved cognitive performance relative to control rats in tasks measuring memory capacity and precision, temporal processing, and attention (Meck et al., 1988; Meck et al., 1989; Zeisel et al., 1991; Meck and Williams, 1997b; Meck and Williams, 1997c; Meck and Williams, 1997a; Ricceri and Berger-Sweeney, 1998;

Williams et al., 1998; Brandner, 2002; Meck et al., 2008). In contrast, prenatally choline deficient rats show a different pattern of cognitive changes as adults. During the acquisition and maintenance of spatial working memory performance on the standard radial-arm maze task, adult prenatal choline deficient rats sometimes show improvements in choice behavior similar to the prenatally choline supplemented rats. However, when the cognitive demands of the task are increased (e.g., by massing trials) dramatic impairments in spatial memory are observed in these rats (Meck and Williams, 1999). Similar improvements and decrements in performance for prenatal choline supplemented and deficient rats, respectively, have been reported for temporal processing tasks that require divided attention or increments in attention following a change in the predictiveness of a stimulus (Meck and Williams, 1997c; Meck and Williams, 1997a; Buhusi et al., 2008; Lamoureux et al., 2008). A recent study revealed that prenatal choline deficiency also produces deficits in sensory inhibition in adult rats (Stevens et al., 2008a), which the authors suggest may contribute to reductions in attentional processes (Meck and Williams, 1997c).

In addition to its memory enhancing effects, prenatal choline supplementation is also remarkably neuroprotective. For example, early choline supplementation confers protection against cognitive and behavioral deficits as a result of several neural insults, including aging, injury, teratogens, and neurological disorders. Prenatal choline supplemented rats are completely protected against age-related declines in spatial memory performance on the radial-arm maze (Meck and Williams, 2003; Meck et al., 2008). Dietary choline supplementation early in development has also been shown to

protect rats from spatial learning and memory deficits normally observed after excitotoxin-induced seizures (Yang et al., 2000; Holmes et al., 2002) and after neonatal alcohol exposure (Thomas et al., 2004; Wagner and Hunt, 2006; Thomas et al., 2007). Reduced birth and brain weight, and alterations in behavioral (e.g., reflex and motor coordination) development in rats neonatally exposed to alcohol were also improved by prenatal choline supplementation (Thomas et al., 2009). Recent studies using mouse models of neurological disorders have also demonstrated that prenatal choline supplementation leads to improvements in sensory inhibition in a model of schizophrenia-like behavior (Stevens et al., 2008b), in motor function in a model of Rett syndrome (Nag and Berger-Sweeney, 2007), and in attention and emotional reactivity in a model of Down syndrome and Alzheimer's disease (Moon et al., 2009). In addition to improved behavioral outcomes in models of injury and disease, prenatal choline supplementation also protects from the amount of neural damage. For example, reduced hippocampal damage after excitotoxic injury (Holmes et al., 2002) and decreased neurotoxicity-induced neurodegeneration of cingulate and retrosplenial cortices (Guo-Ross et al., 2002; Guo-Ross et al., 2003) have been observed in prenatal choline supplemented rats. Prenatal choline supplementation also ameliorates some of the neural deficits associated with models of neurological disorders. Hippocampal nicotinic receptor expression is enhanced by perinatal choline supplementation in a mouse model of schizophrenia-like behavior (Stevens et al., 2008b). Striatal growth factor expression and total brain volume are increased by perinatal choline supplementation in a mouse model of Rett syndrome (Nag et al., 2008; Ward et al., 2008). These choline-induced alterations

in brain may partially underlie the improved behavioral outcomes in these disease models.

In addition to its effects on cognition, altered dietary choline early in life leads to persistent changes in features of adult hippocampal plasticity known to support learning and memory function. Enhanced hippocampal plasticity likely plays a significant role in the memory-enhancing and neuroprotective effects of prenatal choline supplementation. For example, prenatal choline supplementation leads to a reduced stimulus threshold to induce long-term potentiation (LTP) (Pyapali et al., 1998; Jones et al., 1999), increased dendritic spine density in the CA1 and dentate gyrus (Meck et al., 2008), enhanced depolarization-induced phosphorylation of mitogen-activated protein kinase (MAPK) and cAMP-response element binding protein (CREB) activation (Mellott et al., 2004), and enhanced expression of various neurotrophic/growth factors, including brain-derived neurotrophic factor (BDNF) (Glenn et al., 2007), nerve growth factor (NGF) (Sandstrom et al., 2002), vascular endothelial growth factor (VEGF) (Glenn et al., 2007), neurotrophin-3 (NT-3) (Glenn et al., 2007), and insulin-like growth factors- 1 and 2 (IGF-1 and -2) (Napoli et al., 2008; Wong-Goodrich et al., 2008a).

Enhanced hippocampal plasticity and trophic support may also underlie prenatal choline supplementation's neuroprotection of the hippocampus, rendering the hippocampus better able to withstand or recover from the deleterious effects of a neural insult—similar to a young brain. For example, much like prenatal choline supplemented adult rats, juvenile rats that experience prolonged seizures also show resistance to seizure-induced neural damage (Haas et al., 2001) and exhibit savings in spatial learning

and memory compared to adult rats that experience prolonged seizures (Stafstrom et al., 1993; Sarkisian et al., 1997). The retention of juvenile-like neuroplasticity into adulthood may contribute to prenatal choline supplementation's preservation of cognitive function and/or enhanced ability to recover following injury. The same may hold true for the aging hippocampus; enhanced hippocampal plasticity (e.g., proliferation of neural progenitors, growth factor expression) in prenatal choline supplemented rats is retained even as rats age (Glenn et al., 2008b), which may underlie prenatal choline's protection against dramatic declines in spatial memory with age (Meck and Williams, 2003; Meck et al., 2008).

In contrast, prenatal choline deficiency can compromise hippocampal plasticity in adulthood. When compared to control-fed rats, adult offspring of dams depleted of choline on ED12-17 display a higher threshold to induce LTP (Jones et al., 1999), show reduced MAPK and CREB activation (Mellott et al., 2004) and have lower levels of hippocampal IGF-1 (Wong-Goodrich et al., 2008a). A reduced capacity for adult hippocampal plasticity may underlie the cognitive impairments elicited by prenatally choline deficient rats compared to control-fed rats when hippocampal task demands (e.g., massed trials on a radial-arm maze) are increased (Meck and Williams, 1997b; Meck and Williams, 1997c; Meck and Williams, 2003).

Why Neurogenesis?

Recent evidence from our laboratory has now demonstrated that prenatal choline availability also modulates adult hippocampal neurogenesis. Prenatal choline supplementation enhances basal levels of cell proliferation and neurogenesis (i.e., number of immature neurons) in the dentate gyrus of young adult, middle-aged, and aged rats (Glenn et al., 2007; Glenn et al., 2008b; Wong-Goodrich et al., 2008a). However, this work has demonstrated no difference in basal cell proliferation and numbers of immature neurons in the adult dentate gyrus between adult control-fed and prenatally choline deficient rats (Glenn et al., 2007). Rather, prenatally choline deficient rats fail to increase dentate neurogenesis in response to an environmental enriching experience (Glenn et al., 2007). This work has also shown that prenatally choline supplemented rats do not further upregulate hippocampal neurogenesis in response to an enriching experience, while control-fed rats exposed to enrichment upregulate neurogenesis to levels comparable to that of prenatally choline supplemented naïve rats (Glenn et al., 2007), suggesting that the basal neurogenesis condition of prenatally choline supplemented rats may be most favorable for the adult hippocampal network to support optimal learning and memory function.

There are several reasons why adult hippocampal neurogenesis may be one potential mechanism for the effects of altered prenatal choline availability on spatial learning and memory across the lifespan. First, dietary choline supplementation during ED 12-17 increases cell division and neurogenesis as early as ED 16-20 in the fetal mouse and rat hippocampus without altering rates of apoptosis—though relative levels of apoptotic cells are presumed to be less given the increase in neurogenesis (Albright et al., 1999; Craciunescu et al., 2003), while fetuses of dams made deficient of choline on ED12-17 show less cell division and more apoptotic cell death (Albright et al., 1999; Craciunescu et al., 2003). It is thus possible that choline availability during early

development programs the capacity for hippocampal neurogenesis (basal levels and in response to neurogenic stimuli) that persists into adulthood.

Second, because the cholinergic system has a central role in memory function (Bartus et al., 1982; Hasselmo and Giocomo, 2006) and appears to regulate adult hippocampal neurogenesis (Cooper-Kuhn et al., 2004; Mohapel et al., 2005; Kaneko et al., 2006; Kotani et al., 2006), it seems likely that changes in basal forebrain cholinergic function may alter levels of hippocampal neurogenesis to produce changes in spatial memory. Our dietary choline manipulation takes place during the same time period when basal forebrain cholinergic neurons are undergoing neurogenesis (Semba and Fibiger, 1988), which may developmentally program basal forebrain cholinergic function and cholinergic neurotransmission in the adult brain. Indeed, prenatal choline availability produces long-term adaptations in the synthesis, storage and release of acetylcholine, and reuptake and recycling of choline in the adult hippocampus (Blusztajn et al., 1998; Cermak et al., 1998; Cermak et al., 1999; Meck et al., 2008) as well as alterations in the size and shape of basal forebrain cholinergic neurons that project to the hippocampus (Williams et al., 1998; McKeon-O'Malley et al., 2003). Furthermore, neural stem cells, immature neurons, and newborn mature neurons in the adult dentate gyrus express various acetylcholine receptor subtypes, are innervated by cholinergic fibers, and are upregulated by systemic administration of cholinergic agonists (Mohapel et al., 2005; Kaneko et al., 2006; Kotani et al., 2006; Kotani et al., 2008; Itou et al., in press), which suggest that neural stem/progenitor cells and adult-born neurons in the dentate gyrus have the molecular machinery to respond to cholinergic input. Enhancements in adult

cholinergic function prenatally programmed via prenatal choline availability may thus influence levels of adult hippocampal neurogenesis. Notably, olfactory bulb neurogenesis in the adult brain is also regulated by cholinergic input (Cooper-Kuhn et al., 2004; Kaneko et al., 2006) and may thus be similarly modulated by altered prenatal choline availability as that of adult hippocampal neurogenesis. Thus, examining both regions of adult neurogenesis may be useful for exploring whether prenatal choline alters specific populations of stem/progenitor cells, cholinergic modulation of neurogenesis, or both.

Third, previous work has demonstrated that prenatal choline availability alters levels of neurotrophic/growth factors that are both neuroprotective and stimulate adult hippocampal neurogenesis (Sandstrom et al., 2002; Glenn et al., 2007; Glenn et al., 2008b; Wong-Goodrich et al., 2008a; Wong-Goodrich et al., 2008b). It is also possible that prenatal choline availability alters growth factor production in the adult hippocampus to modulate levels of neurogenesis. Prenatal choline supplemented rats show evidence of enhanced cholinergic neurotransmission (Blusztajn et al., 1998; Cermak et al., 1998; Cermak et al., 1999) and increased responsiveness to cholinergic stimulation (Montoya et al., 2000), factors that are known to upregulate hippocampal BDNF and NGF expression (Lindefors et al., 1992; da Penha Berzaghi et al., 1993; Knipper et al., 1994). Prenatal choline supplementation also enhances the phosphorylation of CREB (Mellott et al., 2004), and CREB-related factors regulate BDNF transcription (Tao et al., 1998).

Fourth, a review of the current literature on adult hippocampal neurogenesis suggests that not only is this type of neuroplasticity important for aspects of memory (e.g., Snyder et al., 2005; Winocur et al., 2006; Deng et al., 2009), perhaps because these new neurons are activated and recruited during retrieval of a spatial memory, and this recruitment participates in the strengthening of that memory (Kee et al., 2007; Trouche et al., 2009), but also that hippocampal neurogenesis may to be important under conditions where demands for plasticity are increased (e.g., cognitive task demands, aging, injury). Similarly, prenatal supplementation's enhancing effects on hippocampal learning and memory appear to be most robust under circumstances that impose a challenge to the animal, such as increasing the cognitive demand during memory tasks (Meck and Williams, 1997b; Meck and Williams, 2003; Wong-Goodrich et al., 2008a), in the face of hippocampal aging (Meck and Williams, 1997c; Meck and Williams, 2003; Meck et al., 2008), or after a neuropathological insult (Yang et al., 2000; Holmes et al., 2002; Thomas et al., 2004; Wagner and Hunt, 2006; Thomas et al., 2007). It is possible that prenatal choline supplemented rats that have more new neurons in the adult hippocampus under basal conditions are already functioning optimally when conditions that require greater plasticity occur. This proposed mechanism may explain why supplemented animals show the greatest cognitive advantage when challenged. In contrast, prenatal choline deficient animals are unable to upregulate the number of new dentate granule cells in response to spatial exploration (Glenn et al., 2007) and this deficit in plasticity may explain why these animals in particular show impairments when cognitive task demands are increased.

While it is clear that prenatal choline availability alters levels of hippocampal neurogenesis in the intact brain, it is not clear what step in the process of adult granule cell proliferation and maturation is being altered, or whether prenatal choline availability alters injury-induced neurogenesis. It is also unknown whether prenatal choline availability can modulate levels of stem/progenitor cells or newborn neurons in other neurogenic regions of the adult brain, like the subventricular zone and the olfactory bulb. A major aim of this dissertation research is to answer these questions. The following section is a brief review of granule cell development in the adult hippocampus and a discussion of reactive neurogenesis in the adult hippocampus to both enrichment/learning and neural insult (seizures) that provide the background for the current studies. A brief review of SVZ/olfactory bulb neurogenesis is provided in Chapter 2.

Adult Hippocampal Neurogenesis

The dentate gyrus of the hippocampus is one of two undisputed brain regions known to exhibit adult neurogenesis in the mammalian brain. The other neurogenic region of the adult brain is the subventricular zone (SVZ) of lateral ventricles that gives rise to new neurons in the olfactory bulb (Altman and Das, 1965; Lois and Alvarez-Buylla, 1994; Alvarez-Buylla and Garcia-Verdugo, 2002). Since its first discovery in the adult rat brain in 1965 (Altman and Das, 1965), the presence of hippocampal neurogenesis in the adult dentate gyrus has now been confirmed in many other mammalian species, including mice (Kempermann et al., 1997b; Kempermann et al., 1997a; Kempermann et al., 1998), rabbits (Gueneau et al., 1982), guinea pigs (Guidi et al., 2005), voles (Fowler et al., 2002; Ormerod et al., 2003; Ormerod et al., 2004), nonhuman primates (Gould et al., 1999a; Kornack and Rakic, 1999), and even humans (Eriksson et al., 1998; Knoth et al., 2010). In addition to laboratory animals, adult dentate neurogenesis has also been confirmed in rodents living in the wild (Amrein et al., 2004a; Amrein et al., 2004b). However, much of what we know about the functional properties of newly generated dentate granule cells in the mammalian brain emerges from studies using rodents as subjects. Because it is firmly established that general hippocampal neuroanatomy and function are highly conserved across species (West, 1990; Bunsey and Eichenbaum, 1996), it is likely that neurogenesis in rodents and humans serve similar functions. It is important to note, however, that adult dentate neurogenesis is likely not universal to all mammalian species, as some species of bats appear to lack any expression of adult hippocampal neurogenesis (Amrein et al., 2007).

That the adult hippocampus retains the capacity to generate new neurons throughout the lifespan, and that this feature of adult neuronal plasticity is conserved across several mammalian species raises important questions about its functional relevance: what contribution do these new neurons make to a mature hippocampal circuitry that already expresses robust neuroplasticity? Elucidating the functional role of adult hippocampal neurogenesis is particularly challenging because hippocampal neurogenesis is incredibly dynamic: it is expressed within multiple contexts throughout the lifespan (including early development and in response to injury) and can be modulated by a number of intrinsic and extrinsic (both physiological and environmental) factors. Adding to this challenge is that while adult neurogenesis occurs at moderate levels in the intact hippocampus, it is also triggered at unusually high levels in response to damage-inducing neuropathological insults. Whether or not injury-induced hippocampal neurogenesis helps restore hippocampal function is further complicated by our limited understanding of the functional role of adult dentate neurogenesis in the intact brain and how this ongoing process influences neurogenic responses to injury.



Figure 1. Granule Cell Development in the Adult Hippocampus. Adult dentate granule cell development recapitulates early development

Figure 1 summarizes the developmental events exhibited by adult-born granule cells that were examined in the following studies to characterize the effects of prenatal choline availability on hippocampal neurogenesis. A resident population of proliferating neural stem-like cells are thought to provide the principal source of neurogenesis in the adult dentate gyrus (Kempermann et al., 2004; Suh et al., 2007; Duan et al., 2008; Zhao et al., 2008). These neural stem cells express the Sry-related high-mobility group (HMG) transcription factor Sox-2, a neural stem cell marker that is also expressed by embryonic stem cells neural epithelial cells during development (Zappone et al., 2000; Avilion et al., 2003; Qu and Shi, 2009). It is thought that Sox-2-expressing radial glia-like putative stem cells, termed Type-1 cells, may be the principal stem cell in the adult dentate gyrus (Kempermann et al., 2004; Duan et al., 2008; Zhao et al., 2008). Type-1 cells have radial glia-like morphology with small triangular cell bodies residing in the

subgranular zone (SGZ) and long radial glia-like apical processes extending through the granule cell layer (GCL) (Cameron et al., 1993; Seri et al., 2001; Filippov et al., 2003; Kronenberg et al., 2003; Kempermann et al., 2004; Seri et al., 2004). Type-1 cells also express both the precursor cell division marker nestin and the astrocytic protein glial fibrillary acidic protein (GFAP) (Cameron et al., 1993; Seri et al., 2001; Komitova et al., 2002; Filippov et al., 2003; Fukuda et al., 2003; Garcia et al., 2004; Kempermann et al., 2004; Seri et al., 2004; Liu et al., 2010). These adult neural precursor cells are reminiscent of the stem cells that give rise to the developing hippocampus, as GFAPpositive radial glial cells provide the necessary scaffold for the formation of the dentate gyrus and other regions of hippocampus (Stanfield and Cowan, 1979; Forster et al., 2002; Imura et al., 2003; Anthony et al., 2004). In the adult dentate gyrus, however, Type-1 radial glia-like cells have limited proliferative activity and comprise a very small proportion (< 3%) of the dividing cells in the SGZ (Kronenberg et al., 2003; Suh et al., 2007). The current view (see Duan et al., 2008; Zhao et al., 2008) is that Type-1 cells are multipotent; they divide symmetrically, or asymmetrically and within 24 hours give rise to either astrocytes that express Sox-2, GFAP, and other astrocytic markers (e.g., S100) and that are found mainly in the hilus region with a stellate morphology (Komitova and Eriksson, 2004; Steiner et al., 2004; Duan et al., 2008), or Type-2 neural stem/progenitor cells.

Type-2 cells are transiently amplifying progenitor cells that mark the transition phase between radial glia-like to early post-mitotic and immature neuron phases (Cameron et al., 1993; Kronenberg et al., 2003; Kempermann et al., 2004; Zhao et al., 2008). Recent *in vivo* fate analysis experiments have shown that nonradial Sox-2 expressing Type-2 neural stem cells have the capacity to self-renew and are also multipotent, able to give rise to radial glial-like Type-1 cells, astrocytes, and neuroblasts (Suh et al., 2007). Type-2 cells have unevenly shaped nuclei with dense chromatin, extend short thick processes that spread out in a horizontal fashion, and migrate tangentially (laterally) along the SGZ (Kronenberg et al., 2003; Kempermann et al., 2004). These cells are highly proliferative (accounting for the majority of dividing cells in the SGZ) and continue to express nestin and Sox-2, but do not express GFAP and do not exhibit other astrocytic features characteristic of Type-1 cells (Filippov et al., 2003; Fukuda et al., 2003; Kronenberg et al., 2003; Kempermann et al., 2004). Type-2 cells are also subclassified by their lack of (Type-2a) or presence of (Type-2b) the immature neuronal marker doublecortin (DCX) (Kronenberg et al., 2003), a microtubule associated protein critical for neuronal differentiation, migration, and neurite outgrowth during development (des Portes et al., 1998; Francis et al., 1999).

Both Type-2 cell subtypes undergo cell division and by 3 days after initial cell birth, majority of Type-2 cells mature into Type-3 cells (or neuroblasts) (Kronenberg et al., 2003; Kempermann et al., 2004). Type-3 cells stop expressing nestin and Sox-2, but continue to express the immature neuronal marker DCX (Kronenberg et al., 2003; Kempermann et al., 2004; Komitova and Eriksson, 2004). These neuroblasts have round nuclei, extend longer horizontal and vertical processes, radially migrate a very short distance through the inner granule cell layer, and gradually co-express post-mitotic mature neuronal markers, such as neuronal nuclei (NeuN) (Cameron et al., 1993; Brandt et al., 2003; Brown et al., 2003b; Kempermann et al., 2003; Kempermann et al., 2004; Rao and Shetty, 2004). Type-3 cells are still considered progenitor cells because they undergo an initial period of continued division, though their proliferative activity is quite limited in comparison to Type-2 cells (Kronenberg et al., 2003) and most Type-3 cells will express post-mitotic markers by 3 days after cell birth (Brandt et al., 2003; Kempermann et al., 2003; Kempermann et al., 2004). It is during this time where most cells that do not become recruited into the circuitry rapidly undergo apoptotic cell death (Cameron et al., 1993; Biebl et al., 2000; Seki, 2002; Kempermann et al., 2004). Type-3 cells mark the transition to the post-mitotic immature neuron phase.

During the early post-mitotic phase, immature dentate granule cells start to migrate and as early as 4-10 days after cell division start to extend processes (Stanfield and Trice, 1988; Hastings and Gould, 1999; Markakis and Gage, 1999; Zhao et al., 2008). Continued migration through the GCL during the post-mitotic phase is limited and most immature neurons arrive in the inner third of the GCL as their final destination, suggesting that migration of newborn granule cells is more or less completed shortly after cell division (Kempermann et al., 2003). This phase of DCX expression also encompasses a period of significant dendritic and axonal growth and functional (electrophysiological and synaptic) maturation (Ben-Ari, 2001; Ben-Ari, 2002; van Praag et al., 2002; Ambrogini et al., 2004; Esposito et al., 2005; Overstreet Wadiche et al., 2005; Wang et al., 2005a; Wang et al., 2005b; Karten et al., 2006; for review, see Duan et al., 2008). Immature neurons at this stage have round or slightly triangular nuclei and a clearly visible apical dendrite (Kempermann et al., 2004; Duan et al., 2008; Zhao et al., 2008). As surviving newborn cells start to express markers of neuronal maturity, DCX expression declines dramatically until it becomes undetectable about 4 weeks after initial cell division (Brandt et al., 2003; Brown et al., 2003b; Kempermann et al., 2003; Rao and Shetty, 2004). Upon successful synaptic incorporation into the hippocampal circuitry, adult-born neurons in the dentate gyrus of the adult rodent hippocampus can persist for at least 11 months after cell division (Kempermann et al., 2003).

By the fourth week, the majority of these neurons will adopt a mature morphology, express histological markers of neuronal maturity, and become functionally incorporated into mature neural networks (Jessberger and Kempermann, 2003; Bruel-Jungerman et al., 2006; Ramirez-Amaya et al., 2006; Kee et al., 2007). At 4 weeks, newly generated neurons in the adult dentate gyrus also express activity-dependent immediately early genes (IEG) c-fos, Arc, and Zif268 under behavioral conditions that engage the hippocampus, including spatial learning, spatial navigation, and during the retrieval of a spatial memory (Jessberger and Kempermann, 2003; Bruel-Jungerman et al., 2006; Ramirez-Amaya et al., 2006; Kee et al., 2007; Trouche et al., 2009), which provides further support that these newborn neurons are recruited and actively participate within mature neural networks, and contribute to the updating and strengthening of spatial memory (Trouche et al., 2009). Compared to mature granule cells, newly generated dentate granule cells at 4-6 weeks of age are preferentially recruited during spatial learning (Kee et al., 2007), and also exhibit enhanced synaptic plasticity, including LTP and LTD (Wang et al., 2000; Snyder et al., 2001; van Praag et al., 2002; Ambrogini et al., 2004; Schmidt-Hieber et al., 2004; Bischofberger et al., 2004, as cited in Song et

al., 2005; Ge et al., 2007). One hypothesis is that immature dentate neurons have a certain degree of plasticity that mature dentate neurons lack, so the retention of some "youth-like" properties within the adult hippocampus may allow for better cognitive processing.

In the adult rodent brain, hippocampal neurogenesis levels begin a precipitous decline as early as 7.5-9 months, continue to decline through middle age (12-18 months), and then continue at very low levels into old age (24-27 mo) (Seki and Arai, 1995; Kuhn et al., 1996; Kempermann et al., 1998; Seki and Arai, 1999; Seki, 2002; Bizon and Gallagher, 2003; Rao et al., 2005; Rao et al., 2006). Despite this age-related decline, however, prenatally choline supplemented rats still exhibit more newborn dentate cells than control-fed rats during young adulthood, middle-age, and old age (Glenn et al., 2007; Glenn et al., 2008b; Wong-Goodrich et al., 2008a), which may contribute to lifelong improvements in memory capacity and precision. The following studies examined a number of neurogenic properties discussed that may be potential mechanisms for how prenatal choline alters adult hippocampal neurogenesis, such as mitotic factors, stem cell number or phenotype properties, and/or survival, maturation and neuronal differentiation.

Functional neurogenesis?

Adult-born hippocampal neurons display the physiological properties of neurons, which suggest that they likely make useful contributions to existing neuronal networks. As mentioned previously, accumulating evidence points to a role for hippocampal neurogenesis in spatial discrimination/pattern separation (Clelland et al., 2009), aspects of spatial learning and memory (Madsen et al., 2003; Raber et al., 2004b; Snyder et al., 2005; Winocur et al., 2006; Deng et al., 2009), associative fear memory (Winocur et al., 2006; Kitamura et al., 2009; Snyder et al., 2009), and/or associating events that occur in close temporal proximity (Shors et al., 2001; Shors et al., 2002; Aimone et al., 2006). New evidence suggests that newborn neurons in the hippocampus may also contribute to the decay process of the period during which the hippocampus is required for the expression of long-term memory (Kitamura et al., 2009). However, the functional relevance of hippocampal neurogenesis in the adult brain still remains a somewhat controversial issue. This may be in part due to several factors, including the observation that neurogenesis appears to participate in some, but not all types of hippocampal cognitive function, new evidence that highlights species differences in neurogenesis between mice vs. rats (Snyder et al., 2009), as well as current limitations in the two major approaches that have been used to investigate this issue.

One approach is to experimentally eliminate or severely knock down neurogenesis in the adult hippocampus and then examine the consequences on hippocampal function (ablation). The other approach is to investigate under which conditions adult hippocampal neurogenesis is modulated (reactive neurogenesis), including the influence of both intrinsic (physiological) and extrinsic (e.g., interactions with the physical environment) factors. The type of intrinsic or extrinsic factor that stimulates reactive neurogenesis (proliferation and/or survival and functional integration of new neurons) may be important for determining whether adult dentate neurogenesis makes any contribution to learning and memory. Moreover, the basal condition of adult hippocampal neurogenesis may actually influence properties of neurogenesis that occurs
in response to neurogenic stimuli. The following two potential (though not necessarily mutually exclusive) functional roles of adult dentate neurogenesis have been suggested in the literature (see below). Newborn granule cells are an integral part of a mature hippocampal circuitry: 1) because some conditions demand a greater degree of flexibility that cannot be otherwise compensated for by alternate mechanisms of neural plasticity; and 2) to restore neural and cognitive function under conditions in which hippocampal function becomes compromised. These two possible functional roles are examined in the current studies.

The neurophysiological properties of adult-generated neurons may reflect a critical source of additional plasticity available to the mature hippocampal network. It has been suggested that the contribution of additional synaptic strength by immature granule neurons to the existing hippocampal circuitry may help facilitate more efficient processing under higher cognitive demands (Gould et al., 1999b; Shors et al., 2002; Doetsch, 2003; Aimone et al., 2006; Leuner et al., 2006), such as encoding complex novel stimuli in a changing environment for the assembly of cognitive maps or formation of episodic memories, sustaining an already learned episode within the context of new intervening information, rapid transient storage and erasure of events, or long-term retention of episodic events. In contrast, performing other types of hippocampal functions may only require the contribution of a mature circuitry using other features of plasticity. This explanation would account for why ablation studies reveal that performance on some, but not all, types of hippocampal-dependent tasks is impaired by the elimination of dentate neurogenesis (Shors et al., 2001; Madsen et al., 2003; Raber et al., 2004b; Snyder

et al., 2005; Winocur et al., 2006; Deng et al., 2009). For example, there are number of studies that have directly implicated adult hippocampal neurogenesis in long-term memory retention, while excluding its involvement in spatial learning (Madsen et al., 2003; Raber et al., 2004a; Snyder et al., 2005; Saxe et al., 2006; Winocur et al., 2006; Deng et al., 2009). In this view, while neurogenesis may help enhance performance on hippocampal tasks, neurogenesis becomes critical only when the mature hippocampal circuitry cannot sufficiently meet the demands that are placed upon it.

Neurogenesis in the adult dentate gyrus may also serve a restorative function under conditions where the hippocampus becomes compromised, such as aging or after trauma. Although new neuron production is low in the aged hippocampus, the fact that the aged hippocampus—even at the very end of its lifespan—still has the capacity to generate new neurons at a basal level and in response to stimuli is quite remarkable. Both enrichment (Kempermann et al., 1998) and exercise (van Praag et al., 2005) have been show to increase dentate neurogenesis and reverse spatial learning and memory deficits in aged mice. Though still controversial, injury-induced neurogenesis has also been linked with recovery of cognitive function (Kokaia and Lindvall, 2003; Sun et al., 2005; Lichtenwalner and Parent, 2006; Taupin, 2006; Sun et al., 2007; Wiltrout et al., 2007). The contribution of enhanced plasticity from young dentate neurons may thus help compensate for the losses in plasticity within a mature hippocampal network.

The functional contribution of adult generated granule cells may be dependent upon the context under which it occurs. In the intact brain, adult hippocampal neurogenesis may not necessarily contribute to all types of hippocampally mediated cognition. In addition, the functional role of injury-induced hippocampal neurogenesis may depend on the type of injury. While in some cases increased levels of hippocampal neurogenesis may be beneficial, there is also evidence that excessive neurogenesis may be detrimental to hippocampal function under certain conditions, like seizures (Scharfman and Hen, 2007). It is possible that the basal neurogenic state of the adult hippocampus, which is altered by prenatal choline status, may also influence the quality and functional contribution of reactive dentate cell proliferation/neurogenesis that occurs in response to physiological, environmental, and neuropathological events. The challenge for current researchers is to determine the optimal circumstances to enhance hippocampal neurogenesis for optimal cognitive function.

Reactive neurogenesis in the intact brain

That adult hippocampal neurogenesis can be upregulated also suggests that reactive neurogenesis may serve an adaptive function. If newborn neurons are recruited and actively participate during the encoding of rapidly changing and complex stimuli in novel environments (Kempermann, 2002), the capacity to express a basal level of hippocampal neurogenesis that can quickly upregulate is valuable for an animal living in a complex and rapidly changing environment, such as rats or mice that are constantly evading predators and foraging for food in their natural habitat. Heightened sensitivity to various factors that upregulate neurogenesis may be an adaptive strategy for ongoing preparation for dealing with complex novel environments that would require additional plasticity from newborn granule cells. Levels of adult hippocampal neurogenesis are influenced by a variety of intrinsic (e.g., hormonal alterations, growth factors, aging,

disease) and extrinsic (e.g., exercise, learning experience, environment, brain injury) factors. Using this approach, the modulation of neurogenesis and alterations in learning and memory induced by these intrinsic and extrinsic factors can be correlated. This approach, however, is limited in terms of isolating the specific contribution of dentate neurogenesis to cognition, as identified neurogenic factors also exert effects on other types of hippocampal plasticity important for learning and memory function (e.g., dendritic arbor and spine density, LTP/LTD properties, place cell function) (van Praag et al., 1999a; Poe et al., 2001; Wilson et al., 2004; Eadie et al., 2005; Artola et al., 2006; Olson et al., 2006). So, while factors known to either positively or negatively modulate hippocampal learning and memory function have also been shown to elicit analogous changes in hippocampal neurogenesis, such as environmental enrichment (Kempermann et al., 1997b; Kempermann et al., 1998; Nilsson et al., 1999; Bruel-Jungerman et al., 2005), physical activity/exercise (van Praag et al., 1999a; van Praag et al., 2005), hormones (Cameron and Gould, 1994; Tanapat et al., 1999), stress (Gould and Tanapat, 1999), social isolation (Dong et al., 2004), neurotrophic and growth factors (Shimazu et al., 2006; Zhao et al., 2007), and aging (Kempermann et al., 1998; Kempermann, 2002; van Praag et al., 2005), it is not possible to determine a causal relation between these neurogenic events and cognitive function.

Some neurogenic factors only exert their effects on specific stages of granule cell development. For example, environmental enrichment and spatial learning selectively enhance the survival (as opposed to proliferation) of newborn dentate neurons (Kempermann et al., 1997b; Gould et al., 1999a). This feature of enhanced neurogenesis

has been linked with improved cognitive function, as enrichment also leads to improved performance on learning and memory tasks (Kempermann et al., 1997b; Kempermann et al., 1998; Nilsson et al., 1999; Frick et al., 2003; Bruel-Jungerman et al., 2005). Enrichment paradigms subject an animal to experiences that elicit behaviors that engage the hippocampus, such as exploring novel and familiar objects and locations (O'Keefe, 1979; Eichenbaum, 1999; Kempermann et al., 2004; Lee et al., 2005). Given that newborn neurons must sufficiently mature to become actively recruited for spatial learning (Kee et al., 2007) and express activity-dependent immediate early genes under conditions that elicit hippocampal behaviors (Jessberger and Kempermann, 2003; Ramirez-Amaya et al., 2006; Kee et al., 2007), it is possible that enrichment paradigms enhance survival of newborn neurons by promoting the opportunity for newborn neurons to become actively recruited and express experience-dependent neural activity. Enhancing proliferation of neuronal precursors per se may not necessarily be useful if these newborn neurons are unable to survive and become functionally incorporated into the mature circuitry. Thus, reactive neurogenesis in the adult hippocampus may not necessarily be functionally relevant for spatial learning and memory unless the increase is occurring specifically in the hippocampus, and under conditions that engage the hippocampus. For example, the effect of hippocampal-dependent learning on neurogenesis is specific to the dentate gyrus, as hippocampal learning does not alter neurogenesis in the subventricular zone/olfactory bulb (Gould et al., 1999a). In addition, training on hippocampal-dependent tasks, such as trace eyeblink conditioning and the place version of the Morris water maze, has been shown to enhance the survival of newly

generated dentate neurons that persist for months after training (Gould et al., 1999a; Leuner et al., 2004), whereas training on tasks that do not require the hippocampus, like delay eyeblink conditioning and the striatal-dependent cue version of the Morris water maze, do not elicit changes in hippocampal neurogenesis (Gould et al., 1999a).

Not all neurogenic factors, however, necessarily engage the hippocampus in an experience-dependent manner. For example, genetic, local, or systemic administration of neurotrophic and growth factors, such as brain derived neurotrophic factor, BDNF (Scharfman et al., 2005), NGF (Frielingsdorf et al., 2007), IGF-1 (Aberg et al., 2000; Anderson et al., 2002), FGF-2 (Rai et al., 2007), and VEGF (Jin et al., 2002) to an animal without any changes in its environment will also enhance either proliferation or survival (or both) of newborn dentate granule cells. Although the general assumption is that upregulated neurogenesis is beneficial for learning and memory, it is also possible that the stimulatory or inhibitory effects of some factors on dentate neurogenesis make no direct contribution to cognitive function and are merely secondary artifacts produced by changes in the hippocampal microenvironment. For example, physical exercise is known to upregulate both hippocampal neurogenesis (van Praag et al., 1999a; van Praag et al., 1999b) as well as a number of growth factors, including NGF, BDNF, IGF-1, and FGF-2 (Gomez-Pinilla et al., 1998; Cotman and Berchtold, 2002). Some growth factors appear to be necessary for exercise-induced increases in hippocampal neurogenesis, such as IGF-1 (Trejo et al., 2001; Chen and Russo-Neustadt, 2007) and VEGF (Fabel et al., 2003), and for enrichment-induced neurogenesis, such as BDNF (Rossi et al., 2006).

As previously mentioned, prenatal choline supplemented rats compared to control-fed rats do not appear to further upregulate neurogenesis (which was already heightened in the basal condition) in response to an enriching experience, whereas prenatally choline deficient rats fail to upregulate neurogenesis beyond basal levels that were comparable to that of naïve control-fed rats (Glenn et al., 2007), suggesting that prenatal choline availability alters properties of enrichment-induced neurogenesis in the intact hippocampus. The present studies, therefore, attempted to examine the potential mechanisms underlying the lack of enrichment-induced neurogenesis expressed by prenatally choline supplemented (perhaps due to a ceiling) and deficient (perhaps due to a failure to appropriately respond to neurogenic signals) rats.

Reactive neurogenesis in response to injury

In addition to upregulating in response to changes in the environment or a physiological stimulus, adult hippocampal neurogenesis is also stimulated in response to brain injury. Compared to physiological levels of reactive neurogenesis that occur in the intact hippocampus, levels of hippocampal neurogenesis are dramatically upregulated, perhaps aberrantly, following a neuropathological insult, such as prolonged seizures, ischemia/stroke, and traumatic brain injury. It has been suggested that this robust neurogenic response to brain injury may, after some types of injury (e.g., stroke, traumatic brain injury), be clinically beneficial and interventions to increase levels of neurogenesis following injury have been advocated in many research circles (Kokaia and Lindvall, 2003; Sun et al., 2005; Lichtenwalner and Parent, 2006; Taupin, 2006; Sun et al., 2007; Wiltrout et al., 2007). While this may be true for some brain regions under

some pathological conditions, recent research findings have questioned whether heightened levels of dentate neurogenesis is beneficial for hippocampal function in the context of neural assault, particularly after seizures. For this reason and because this dissertation project focused on the seizure-induced changes in hippocampus, this background will address the changes in hippocampal neurogenesis that occur following seizures.

Status epilepticus (SE), a period of prolonged seizures, produces a host of degenerative and regenerative changes in the hippocampus that are thought to contribute to the development of temporal lobe epilepsy (Obenaus et al., 1993; Houser and Esclapez, 1996; Esclapez and Houser, 1999; Fujikawa et al., 2000; Shetty and Turner, 2001; Kotloski et al., 2002; Parent, 2002). SE can be experimentally induced via chemoconvulsants (e.g., pilocarpine, kainic acid) or sustained electrical stimulation of the hippocampus or amygdala (reviewed in Bernardino et al., 2005). After the acute period of experimentally-induced SE, a silent phase occurs during which no behavioral motor seizures are evident, followed by a chronic epileptic phase characterized by spontaneous recurrent motor seizures (Hellier et al., 1998; Hellier and Dudek, 1999; Loscher, 2002; Hattiangady et al., 2004). During this silent period, a transient surge in neuronal proliferation in the dentate gyrus occurs, beginning after a latent period of at least 2-3 days, peaking during 1-4 weeks, and then returning to basal levels by approximately one month after SE (Bengzon et al., 1997; Parent et al., 1997; Gray and Sundstrom, 1998; Parent et al., 1999; Covolan et al., 2000; Hattiangady et al., 2004). Seizure-induced dentate neurogenesis can occur in the absence of neuronal cell death, suggesting that this

reactive neurogenesis is not a residual product of seizure-induced cell death *per se*, and appears to be positively related to seizure severity (Parent et al., 1997; Parent et al., 1998; Tooyama et al., 2002; Mohapel et al., 2004; Smith et al., 2005). Following this transient period of upregulation and subsequent return to baseline levels, a long-term suppression of dentate neurogenesis eventually occurs during the epileptic phase, weeks to months after SE (Hattiangady et al., 2004; Hattiangady and Shetty, 2010). The functional relevance of these disparate neurogenic responses following seizures to cognitive function is complicated by the fact that deficits in spatial learning and memory are evident both shortly after and months following SE (Stafstrom et al., 1993; Liu et al., 1994; Sutula et al., 1995; Sarkisian et al., 1997; Hort et al., 1999; Yang et al., 2000; Mikati et al., 2001; Wu et al., 2001; Holmes et al., 2002).

More is known about the properties of injury-induced dentate neurogenesis following seizures than any other insult. The transient increase in neuronal proliferation in the dentate gyrus following seizures is a product of enhanced cell division of both radial glia-like (Type-1) precursor cells (Huttmann et al., 2003; Zhu et al., 2005; Steiner et al., 2008, but see also Jessberger et al., 2005), as well as newly differentiated (Type-3) DCX-positive neurons (Hattiangady et al., 2004; Jessberger et al., 2005). Most of the newly generated progenitor cells within the dentate gyrus born in response to seizures survive, differentiate into neurons, and migrate through the GCL (Parent, 2002). There is evidence that the morphology, migration, dendritic growth, and synaptic integration of dentate granule cells born after status epilepticus is altered (Parent et al., 1997; Scharfman et al., 2000; Hattiangady et al., 2004; Overstreet-Wadiche et al., 2006). Many newly generated neurons born after seizures migrate into the dentate hilus and are termed "ectopic granule cells" (Parent et al., 1997; Scharfman et al., 2000; Hattiangady et al., 2004; Scharfman, 2004). These ectopic granule cells are hyperexcitable and have disrupted GABAergic function (Ribak et al., 2000; Scharfman et al., 2000; Dashtipour et al., 2001; Pierce et al., 2005), which may be a component to the recurrent excitatory circuitry that underlies the formation of spontaneous motor seizures in models of temporal lobe epilepsy (Scharfman, 2004; Scharfman and Gray, 2007).

Whether or not these ectopic hilar granule cells contribute to epileptogenesis is still controversial because other plastic changes in the hippocampus following prolonged seizures may also underlie epileptogenesis, such as reactive gliosis (Jorgensen et al., 1993; Niquet et al., 1994a; Niquet et al., 1994b; Kang et al., 2006) and mossy fiber innervation of the dentate gyrus by mature granule cells (Sutula et al., 1988; Ben-Ari and Represa, 1990). Dentate granule cells born after SE that do not aberrantly migrate, however, may actually serve a restorative function, as these cells display lower excitatory synaptic currents (fewer spontaneous EPSCs), a lower probability of glutamate release, enhanced inhibitory drive (more spontaneous IPSCs), and a higher probability of GABA release when compared to dentate granule cells born in the intact hippocampus (Jakubs et al., 2006). The reduced excitability of newborn dentate granule cells in the GCL may thus be beneficial in counteracting the hyperexcitability that underlies epilepsy, which again suggests that it is the aberrant migration that is detrimental while some aspects of seizureinduced neurogenesis may be restorative.

The issue of whether excessive neurogenesis shortly after seizures has any functional consequences for cognitive ability has not been adequately explored, as most studies focus on how dentate neurogenesis and the factors that potentially modulate postseizure neurogenesis contribute to seizure severity during SE and epileptogenesis following SE (Liu et al., 1993; Liu and Holmes, 1997; Gomez-Pinilla et al., 1998; Reibel et al., 2000; Cotman and Berchtold, 2002; Setkowicz and Mazur, 2006). SE-induced seizures lead to profound deficits in hippocampal-dependent learning and memory both shortly after and weeks following seizures (Stafstrom et al., 1993; Liu et al., 1994; Sutula et al., 1995; Sarkisian et al., 1997; Hort et al., 1999; Yang et al., 2000; Mikati et al., 2001; Wu et al., 2001; Holmes et al., 2002). It has been suggested that aberrant changes in hippocampal circuitry as a result of seizure-induced neurogenesis may partially underlie cognitive deficits that arise following seizures, as aberrant connections within new circuits may disrupt normal synaptic function and in turn affect learning and memory processes (Scharfman, 2004). Some treatments can ameliorate learning and memory deficits shortly after seizures, such as prenatal choline supplementation (Yang et al., 2000; Holmes et al., 2002) and voluntary exercise (Gobbo and O'Mara, 2005), although whether these treatments alter properties of seizure-induced dentate neurogenesis is not fully understood and was thus a significant aim of this dissertation research.

While it is not clear whether aberrantly high levels of dentate neurogenesis directly contribute to cognitive deficits observed shortly after SE, it has been suggested that the subsequent persistent downregulation of dentate neurogenesis weeks to months following SE may contribute to cognitive deficits associated with epilepsy (Mikati et al., 2001; Elger et al., 2004; Hattiangady et al., 2004; Hattiangady and Shetty, 2010). Although direct evidence for a causal link is still lacking, this possibility is supported by clinical studies that report a greater severity of memory deficits in patients with a longer duration of epilepsy (Jokeit and Ebner, 1999; Alessio et al., 2004). Diminished dentate neurogenesis during this phase has also been associated with marked reductions in growth factors known to promote neurogenesis, such as IGF-1 and FGF-2 (Hattiangady et al., 2004). In sum, the functional implications of seizure-induced dentate neurogenesis may depend on the migratory pattern of newly generated dentate granule cells born after seizures and on the immediate vs. long-term phase of recovery from SE. It has been suggested that interventions that aim to prevent altogether (or at least the aberrant properties of) seizure-induced dentate neurogenesis while enhancing neurogenesis during the chronic phase may be key to precluding epileptogenesis and restoring cognitive function after SE (Scharfman and Hen, 2007; Arida et al., 2008; Dhanushkodi and Shetty, 2008; Hattiangady and Shetty, 2008b).

Overview

The overarching goal of this dissertation work was to investigate how the availability of a single nutrient, choline, during a specific period during early development alters properties of neurogenesis in the adult brain (in the intact brain and after seizures) and modulates the hippocampal response to and recovery from seizures, and finally, attempt to relate changes in neurogenesis to changes in spatial learning and memory. In the current experiments, the term neurogenesis (the addition of new neurons) is used to encompass both the birth and survival of newborn neurons, and distinctions

between effects on cell proliferation versus survival are made where appropriate. Moreover, while it is noted that the cognitive measures examined in this dissertation research are correlative, these experiments provide a first step towards designing more specific research questions that may potentially be addressed using an ablation approach. Experiments using both intact and injured young adult animals provided the foundation for this dissertation research to address the following questions: 1) what are the proximate mechanisms underlying how prenatal choline availability alters adult hippocampal neurogenesis in the intact brain? 2) Are prenatal choline's effects on levels of basal neurogenesis in the intact adult brain specific to the hippocampus? 3) Does altered prenatal choline availability modify the short- and/or long-term effects of excitotoxic injury (seizures) on dentate cell proliferation/neurogenesis and other markers in the hippocampus that contribute to the neuropathological response to seizures? 4) Does enhanced hippocampal plasticity and trophic support conferred by prenatal choline supplementation lead to an altered hippocampal response to seizures that is more favorable for long-term cognitive recovery?

Experiment 1 (Chapter 1) was informed by recent studies from our laboratory that demonstrated that prenatal choline supplementation enhances cell proliferation in the adult dentate gyrus throughout life (Glenn et al., 2007; Glenn et al., 2008b; Wong-Goodrich et al., 2008a), with one study confirming that this increase was observed in numbers of DCX+ immature neurons (Glenn et al., 2007). Experiment 1 sought to further understand this phenomenon and examined whether modulations of adult hippocampal neurogenesis in the intact brain by prenatal choline availability (supplementation and

deficiency) are due to changes in cell division, survival of newborn cells, proportion of newly generated cells that survive, mature, and commit to a neuronal vs. glial phenotype, and/or in the number and proliferative capacity of neural stem cells in the adult dentate gyrus, and whether these changes in neurogenesis are accompanied by alterations in hippocampal neurotrophic factor content. Animals in Experiment 1 were also tested in a delayed matching-to-place version of the water maze to assess spatial memory function, as newborn dentate granule cells have been shown to be important for spatial memory retention (Snyder et al., 2005; Deng et al., 2009) and memory retention in delay matching-to-sample tasks (Winocur et al., 2006), and evaluate whether numbers of surviving newborn neurons correlate with spatial memory performance within individual rats. The design of Experiment 1 also attempted to further elucidate the potential mechanisms underlying why prenatal choline supplemented and deficient rats do not upregulate levels of hippocampal neurogenesis in response to daily spatial exploration (Glenn et al., 2007). A 10 min daily spatial exploration experience using an open arena with toys was used to engage the hippocampus (Jenkins et al., 2004; Nitz and McNaughton, 2004; Ramirez-Amaya et al., 2006; Cheng and Frank, 2008).

Experiment 2 (Chapter 2) also addressed the issue of mechanism by examining whether another population of newborn cells in another neurogenic region of the adult brain is also influenced by prenatal choline supplementation or deficiency. Given that basal forebrain cholinergic input appears to regulate both hippocampal and olfactory bulb neurogenesis (Cooper-Kuhn et al., 2004; Kaneko et al., 2006) and that prenatal choline availability alters the size and shape of basal forebrain cholinergic neurons that project to the hippocampus (Williams et al., 1998; McKeon-O'Malley et al., 2003), it is possible that prenatal choline's modulation of hippocampal plasticity may also affect similar types of neuroplasticity in other neural regions that receive cholinergic input. Indeed, newborn olfactory bulb neurons in the olfactory bulb, but not newborn cells in the SVZ, make contact with cholinergic fibers and express multiple acetylcholine receptor subunits (Kaneko et al., 2006). However, some neurogenic factors exert their effects on adult neurogenesis with regional specificity. For example, environmental enrichment, voluntary wheel running, and hippocampal-dependent spatial learning all selectively enhance neurogenesis in the adult hippocampus, but do not alter cell proliferation in the SVZ or neurogenesis in the olfactory bulb (Gould et al., 1999a; Brown et al., 2003a). Experiment 2 investigated whether prenatal choline availability alters basal levels of SVZ cell proliferation, olfactory bulb neurogenesis, and olfactory bulb neurotrophic factor content in the adult brain in a manner similar to that which has been observed in the adult hippocampus.

Prenatally choline supplemented and deficient rats do not appear to upregulate dentate cell proliferation and neurogenesis in response to a daily goal-directed spatial exploration on a radial arm maze (Glenn et al., 2007), perhaps because supplemented rats are operating at a ceiling and deficient rats are unable to upregulate and/or respond to neurogenic factors that are crucial for reactive neurogenesis. Is this true for all types of reactive neurogenesis? Using a model of excitotoxic injury, Experiment 3 (Chapter 3) and Experiment 4 (Chapter 4) investigated whether a similar pattern emerges when the extrinsic neurogenic factor is pathological in nature and is, in the case of seizures, known

to stimulate a more robust proliferative response the rat dentate gyrus (Bengzon et al., 1997; Parent et al., 1997; Hattiangady et al., 2004). Experiment 3 investigated the effects of prenatal choline *supplementation* on seizure-induced dentate cell

proliferation/neurogenesis that occurred up to 16 days after injury, as well as other known markers in the hippocampus that contribute to the neuropathological response to seizures. It is during this time window where prenatal choline supplementation's protection against seizure-induced spatial learning and memory deficits has been observed (Yang et al., 2000; Holmes et al., 2002). Experiment 4 utilized this same timeline to examine whether prenatal choline *deficiency* alters the proliferative response observed in the dentate gyrus shortly after seizures.

Although previous studies have reported cognitive benefits of prenatal choline supplementation shortly after seizures (Yang et al., 2000; Holmes et al., 2002), whether prenatal choline supplementation confers protection against the long-term effects of seizures on hippocampal function is not known. Moreover, while both prenatal choline supplementation and enrichment/exercise increase hippocampal plasticity and aid recovery from hippocampal injury (e.g., Yang et al., 2000; Holmes et al., 2002; Dhanushkodi and Shetty, 2008; Hattiangady and Shetty, 2008b; Arida et al., 2009), no study has directly compared the effects of these two manipulations on brain and cognitive function to determine if there are similar mechanisms underlying these effects. To examine these issues, Experiment 5 (Chapter 5) determined whether prenatal choline availability and/or repeated water maze training (a form of cognitive enrichment with an exercise component) alters the long-term survival and migration of adult dentate granule cells born shortly after seizures, as well as levels of hippocampal neurogenesis and markers of neuropathology observed in the hippocampus months after excitotoxic injury (when levels of hippocampal neurogenesis are significantly decreased). Importantly, because prenatal choline supplementation enhances hippocampal plasticity and neurotrophic/growth factor expression in the intact adult brain (e.g., Sandstrom et al., 2002; Mellott et al., 2004; Glenn et al., 2007), Experiment 5 asked whether this enhanced hippocampal plasticity and trophic support conferred by prenatal choline supplementation lead to an altered hippocampal recovery from seizures that facilitated the recovery of spatial learning and long-term memory retention in the water maze. Of interest was also whether this enhanced plasticity prior to injury would better enable the adult injured hippocampus to take advantage of the rehabilitative effects of repeated water maze training after seizures.

EXPERIMENT 1: PROXIMATE MECHANISMS UNDERLYING THE EFFECTS OF ALTERED PRENATAL CHOLINE AVAILABILITY ON ADULT HIPPOCAMPAL NEUROGENESIS

Prenatal choline supplementation during embryonic days (ED) 12-17 increases basal levels of adult hippocampal neurogenesis throughout life (Glenn et al., 2007; Glenn et al., 2008b; Wong-Goodrich et al., 2008a). Three weeks of daily spatial exploration on a radial-arm maze does not further upregulate neurogenesis in prenatally choline supplemented rats (which are already showing higher basal levels), or upregulate neurogenesis in prenatally choline deficient rats (which show basal levels of neurogenesis indistinguishable from control-fed rats), despite increasing adult hippocampal neurogenesis nearly 1.5-fold in control-fed rats (Glenn et al., 2007). These data suggest that both basal and reactive neurogenesis in the adult hippocampus are altered by prenatal choline availability.

Experiment 1 was designed to more fully understand the proximate mechanisms underlying the changes in adult hippocampal neurogenesis that were programmed by choline availability (supplementation and deficiency) during ED 12-17. To examine this issue, Experiment 1 determined whether modulations of adult neurogenesis in the dentate gyrus by prenatal choline status are due to changes in: 1) dentate cell division and/or survival, 2) neuronal and glial differentiation, and/or 3) the number, phenotype, and/or proliferative capacity of neural stem/progenitor cells in the SGZ, which provide the principal source of new neurons in the adult dentate gyrus (Garcia et al., 2004; Komitova and Eriksson, 2004; Steiner et al., 2006; Suh et al., 2007). Experiment 1 also examined hippocampal expression levels of brain-derived neurotrophic factor (BDNF) and nerve

growth factor (NGF), neurotrophic factors known to promote adult hippocampal neurogenesis (Lee et al., 2002a; Sairanen et al., 2005; Frielingsdorf et al., 2007) and that have been reliably shown to be upregulated by prenatal choline supplementation in the adult rat hippocampus (Sandstrom et al., 2002; Glenn et al., 2007). To further understand the effects of prenatal choline availability on the mechanisms that govern reactive neurogenesis in the intact adult hippocampus, a spatial exploration task was used in the current experiment to provide a type of enriching experience known to engage various subfields of the hippocampus, including the dentate gyrus (Jenkins et al., 2004; Nitz and McNaughton, 2004; Cheng and Frank, 2008) and newborn granule cells within the dentate gyrus (Ramirez-Amaya et al., 2006).

To investigate whether prenatal choline's modulation of cell proliferation in the adult dentate gyrus can be attributed to changes in the number, phenotype (Type-1/radial glial cells vs. Type-2 non-radial cells), and/or mitotic status of neural stem/progenitor cells in the SGZ, Experiment 1 employed immunostaining for Sox-2, a good marker for identifying neural stem cells in the SGZ of the adult dentate gyrus (Komitova and Eriksson, 2004), in combination with other markers of radial glia, astrocytes, and the endogenous cell division marker, Ki-67 (Scholzen and Gerdes, 2000). One hypothesis is that increased prenatal choline availability may program the developing dentate gyrus such that there is a larger pool of neural stem/progenitor cells throughout life, leading to increased hippocampal neurogenesis that persists into old age (Glenn et al., 2008b). A second, but not mutually exclusive hypothesis is that prenatal choline availability has no

effect on total neural stem/progenitor cell number, but that these stem/progenitor cells are more proliferative in choline supplemented rats.

It is also possible that prenatal choline availability alters the survival of newborn cells in the adult dentate gyrus because it is ultimately the survival and integration of newborn neurons into the existing circuitry that is essential if these newborn neurons are to make any functional contribution to the hippocampal network for learning and memory. Although a neuronal versus glial phenotype is determined within 1-3 days after cell birth in the adult dentate gyrus (Duan et al., 2008), a 7-week cell survival time point was chosen in the current experiment because it captures the full extent of cell death that occurs after BrdU administration (Dayer et al., 2003) and the time point when newborn neurons are functionally integrated into mature hippocampal circuits. By 4-6 weeks after cell birth, newly generated granule cells can express activity-dependent immediately early genes (Jessberger and Kempermann, 2003; Bruel-Jungerman et al., 2006; Ramirez-Amaya et al., 2006; Kee et al., 2007; Tashiro et al., 2007), as well as markers of neuronal maturity (Cameron et al., 1993; Snyder et al., 2009).

Because I am hypothesizing that the enhanced adult hippocampal neurogenesis observed in prenatally choline supplemented rat may, at least in part, contribute to the lifelong enhancements in memory precision and capacity seen in adult and aging rats capacity (Meck and Williams, 2003; Meck et al., 2008), I employed a delayed-matchingto-place (DMTP) water maze task (administered ~5 weeks after BrdU administration) to

assess spatial working memory ability in attempt to relate changes in neurogenesis to changes in memory performance.

Methods

Animals

Forty-three timed-pregnant Sprague-Dawley rats (CD strain, Charles River, Kingston, NY) were obtained on day 9 of gestation (ED9). All dams were individually housed in clear polycarbonate cages $(27.9 \times 27.9 \times 17.8 \text{ cm})$ that were individually ventilated, and the colony was maintained at 21°C on a 12-h light/dark cycle with lights on at 7 a.m. Dams were fed a control diet ad libitum (AIN76-A from Dyets, American Institute of Nutrition, ICN, Nutritional Biochemical, Cleveland, Ohio; 1.1 g/kg choline chloride substituted for choline bitartrate). Prenatal diet treatments were the same as those used in studies showing memory enhancing and memory protecting effects of prenatal choline supplementation (Meck et al., 1988, 1989; Yang et al., 2000; Holmes et al., 2002; Meck & Williams, 2003; Wong-Goodrich et al., 2008a; 2008b). On the morning of ED11 to the morning of ED18 (ED 11-17), pregnant dams were given continued *ad libitum* access to the AIN76-A control diet (n = 17), or were given either a choline supplemented diet (n = 13) or a diet completely deficient of choline (n = 13). Choline supplemented dams received a version of the control diet that contained 5 g/kg choline chloride (approximately 4.5 times more choline than the control diet). Choline deficient dams were given a version of the control diet that was completely deficient of choline. On ED18, all dams were returned to the control diet. There were no significant differences in the amount of food consumed or body weights on ED11-18 between any

diet group of dams (ps > 0.05; data not shown). After birth, offspring from the control, choline supplemented, and choline deficient dams were toe clipped for identification and then were selected randomly and cross-fostered to dams that consumed the control diet throughout pregnancy to yield 10 pups per litter (5 males and 5 females, half from different control dams and half from supplemented dams). There were no significant differences between diet groups in litter size or pup birth weights (ps > .05). On postnatal day (PD) 25, pups were weaned and pair-housed with a rat of the same sex and prenatal diet condition. All offspring were given ad libitum access to the control diet through the duration of the study. Female offspring were used as subjects to be consistent with the previous study (Glenn et al., 2007). All animal procedures were in compliance with the Institutional Animal Care and Use Committee of Duke University.

Timeline of experimental procedures

At approximately 7 months of age, all rats were handled once daily for 3 days prior to the start of the experiment. This age was chosen to be consistent with the previous study first demonstrating the effects of prenatal choline availability on adult hippocampal neurogenesis rats (Glenn et al., 2007). All behavioral procedures took place during the dark phase of the 12/12-hour light-dark cycle (i.e., when the animals were more active). All rats were then given 5 days of daily 5 min sessions of habituation in a spatial exploration arena (see description below) devoid of objects and a curtain pulled around the arena to eliminate extramaze cues. Immediately following the habituation session on each day, vaginal smears were taken from rats to verify that all rats were cycling normally. After 5 days of arena habituation, rats were given 4 daily injections of the mitotic marker bromodeoxyuridine (BrdU) to label dividing cells. Twenty-four hours later (Immediate), a group of rats that were in their estrus phase (confirmed by vaginal cell cytology) from each prenatal diet condition were sacrificed to examine the addition of new cells that occurred over 4 days of BrdU administration (SUP, n = 7; CON, n = 8; DEF, n = 8). The remaining rats were divided into two groups to assess long-term cell survival: one group remained in their home cage with their cagemate for 5 weeks (Home Cage; SUP, n = 10; CON, n = 8; DEF, n = 10), and one group received daily 10 min. sessions of novel spatial exploration for 5 weeks (Experience; SUP, n = 10; CON, n = 10; DEF, n = 10). All Home Cage and Experience rats were then subjected to 10 days of water maze testing, and then sacrificed as soon as each rat entered their estrus phase (vaginal smears were taken to confirm phase of estrus cycle), at approximately 7 weeks after the last BrdU injection. Figure 2 summarizes the timeline of procedures.



Figure 2. Experiment 1 Timeline of Experimental Procedures.

Vaginal smears

The estrous cycle was monitored in each female rat to ensure that all rats were sacrificed during estrus to control for estrous cycle-dependent variations in brain measures. To track each phase of the 4-day estrous cycle in female rats, vaginal smears were obtained to monitor changes in vaginal cell cytology across each phase (proestrus, estrus, metestrus, and diestrus). A sterile cotton tip applicator was used to take a sample of cells from the vaginal walls of each rat. Cell samples were mounted on clean glass slides and examined under a light microscope using a 10x objective. A proestrus smear mainly consists of nucleated epithelial cells; an estrous smear mainly consists of larger, anucleated cornified cells, usually densely clustered together; a metestrus smear consists of a similar proportion of small leukocytes, large anucleated cornified cells, and nucleated epithelial cells; and a diestrus smear primarily consists of leukocytes (Marcondes et al., 2002).

Bromodeoxyuridine injections

One day after arena habituation, all rats were administered a total of 4 daily injections of 5-bromo-2-deoxyuridine (BrdU; 100mg/kg/day, i.p.; Sigma, St. Louis, MO) to label dividing cells. BrdU injections were administered at the same time every day during the dark phase of the light-dark cycle. This injection regimen was chosen to capture each phase of the 4-day estrous cycle because levels of adult dentate cell proliferation/neurogenesis vary across different hormonal-dependent phases of the rat estrous cycle (Tanapat et al., 1999).

Spatial arena habituation and exploration

The exploration arena consisted of an open 60 x 60 cm box with 20 cm high walls placed in the center of a large well-lit room with large extramaze cues present throughout the room (e.g., desks, ladders, posters hanging from ceiling, pictures on wall, bookshelves, computer, and chairs). Prior to BrdU injections, all rats were placed in the arena with their cagemate once per day for 5 days. During this habituation period, the arena contained no objects and a curtain was pulled around the arena to eliminate any extramaze cues.

Starting one day after the last day of BrdU injections, Experience rats were given daily 10 min sessions of novel spatial exploration, which provided a type of enriching experience known to engage various subfields of the hippocampus, including the dentate gyrus (Jenkins et al., 2004; Nitz and McNaughton, 2004; Cheng and Frank, 2008) and, more specifically, newborn granule cells within the dentate gyrus (Ramirez-Amaya et al., 2006).

During the spatial exploration task, various objects (e.g., heavy objects that can be crawled in or on, toys that can be moved) were placed in novel locations throughout the arena (the same arena that was used during habituation) and food pellets were scattered to encourage exploration. Each day, extramaze cues were rearranged throughout the room and a different set of objects was placed in the arena so that rats did not interact with the same object it had seen at least 3 days prior. The room had large hanging curtains on tracks such that the geometry of the room (circular, square, rectangle) could also be changed easily. Varying the spatial arrangements of familiar visual stimuli in particular

has been found to engage subfields of the hippocampus, including the dentate gyrus (Jenkins et al., 2004).

All Experience rats were placed in the exploration arena with their cagemate for 10 min per day for 5 weeks during the first half of the dark phase of the rats' cycle, the time of day of greatest exploration/feeding. The rats' behavior during the exploration task was recorded with a video camera for the first 3 days of exploration and then once per week in the middle of the week for the remaining 4 weeks. The number of contacts with objects, total time spent exploring objects, and the mean time spent exploring a given object were recorded for each rat. This exploration task is similar to that of our prior work using the radial-arm maze (Glenn et al., 2007), but the spatial exploration arena with toys was substituted for the maze to decrease the levels of stress that may ensue from walking out to the end of the maze arms.

Water maze apparatus, design, and behavioral testing procedures

After 5 weeks of daily novel exploration or remaining in the home cage, rats were behaviorally trained for 11 days on a delayed-matching-to-place (DMTP) version of the water maze: 2 days of maze habituation, 5 days of pretraining, 3 days of delay testing, and 1 day to confirm that rats were relying on extramaze cues to navigate the maze. Procedures were adapted from previous studies (Sandstrom et al., 2001; 2004; Wong-Goodrich et al., 2008). The maze consisted of a circular pool approximately 1.8 m in diameter and filled with room temperature water. A circular platform 10 cm in diameter was submerged 2 cm below the surface of the water, and the water was clouded by nontoxic powered tempera paint to ensure that the rats could not see the platform. The pool was located in a well-lit room (approximately $5.8 \text{ m} \times 2.6 \text{ m}$ in dimension) with salient extramaze cues, such as a table with a computer, shelving that contained large objects, pictures of large black shapes adhered to a curtain and room walls, a large metal trash bin, and the experimenter who sat in a chair near the computer. All rats being tested were first habituated to the room and water maze procedures for 2 days. During habituation, rats were first placed on a hidden platform in the middle of the maze for ~10 s, then placed in the water near the hidden platform and gently guided toward the platform.

During pretraining and delay testing, rats were given 2 sessions each day where rats had to locate a hidden platform that was positioned in a different pool location for each session. The first trial in each session served as a train trial where a rat learned the novel platform location. With the exception of the first 2 days of pretraining, the second trial in each pair served as the test trial where a rat was tasked with locating the platform location it had been exposed to on the preceding train trial; on the first 2 days of pretraining, rats received 2 train trials. The duration of the retention interval between trials within a session was varied to assess the effects of increasing cognitive load on working memory. During the retention interval, rats were placed in a holding cage lined with a paper towel within the same testing room. Between each session, rats were dried with a towel and placed back in their home cage with their cagemate and standard bedding, which was outside of the testing room (to signal the end of the session), for approximately 2 hours until the next session commenced. Different start locations were used for each session within a day. Platform and start locations were randomly assigned to each session.

Pretraining procedures were designed to gradually familiarize the rats to the testing procedures that the rats would be exposed to during delay testing, and to provide them with the opportunity to learn how to navigate the maze in order to locate a hidden platform upon which they could escape the cool water. During 5 days of pretraining, all rats were given a 5 min retention delay between train and test trials during each session. On each train and test trial for days 1 - 3 of pretraining, rats were placed in the pool along the edge from one of 8 start locations around the pool and allowed to swim freely until the platform by the end of 60 s, it was led to the platform. Rats were allowed to sit on the platform for 15 s after climbing on to it. On days 4 and 5, testing procedures were identical with the exception of the train trial where rats were not allowed to swim in the pool; instead, rats were placed directly on the platform for 30 s.

During the 3 days of delay testing, rats were subjected to 2 sessions per day where they experienced a 5 min retention delay during one session and a 90 min retention delay during the other session (delay order was counterbalanced across days and across rats). Rats were placed directly on the platform for 30 s during train trials; thus, the train trial experience was held constant for all rats. During the test trials, rats were placed in the pool in a random start location and were allowed to swim throughout the pool to locate the hidden platform. If the rat did not locate the platform within 60 s, it was gently guided to the platform and allowed to sit on the platform for 15 s. On the last day of testing, rats were given one session to assess their use of extramaze relational cues. On the train trial, rats were placed directly on a novel platform location for 30 s presenting the usual fashion. During the test trial after a 5 min retention delay, however, a curtain was pulled around the maze to obscure all extramaze cues. One day after the last day of water maze testing, vaginal smears were collected each day and all rats were sacrificed during their estrus phase (confirmed by vaginal cell cytology) between 2 and 5 days after the final day of water maze testing (~7 weeks after the last BrdU injection).

Performance on the task was recorded, including latency (s), pathlength (m), and swim speed (m/s) to locate the hidden platform, using a computerized tracking system (HVS Image).

Tissue harvesting

Twenty-four hours or 7 weeks after the last BrdU injection, rats were anesthetized with isoflurane, decapitated, and brains were rapidly removed and midsagitally sectioned. The hippocampus from one half-brain from each rat was immediately dissected for growth factor protein assays and stored at -80° C. The remaining half-brain was immediately post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 72 hours at 4° C and then cryoprotected in a 30% sucrose solution in 0.1 M PB. These half-brains were then sectioned coronally at 60 μ m on a freezing microtome through the rostral-caudal extent of the hippocampus and every fifth section was collected in 0.1% sodium azide in 1M PB to yield five series of sections. One series of sections for all rats was processed for BrdU immunohistochemistry, and one series was processed for Sox-2 immunohistochemistry for subsequent cell counting. In a subgroup of rats from each prenatal diet group (*n* = 4), another series was processed for BrdU/NeuN/GFAP triple

immunofluorescence and representative sections were processed for Sox-2/GFAP/S100β triple immunofluorescence for subsequent confocal analysis.

BrdU and Sox-2 immunohistochemistry

Immunohistochemical procedures for BrdU-labeling were based our previous reports (Glenn et al., 2007; Wong-Goodrich et al., 2008a). For BrdU labeling, freefloating sections were rinsed with tris-buffered saline (TBS: pH 7.3) followed by 30 min in 50% methanol and 30 min in 3% hydrogen peroxide in TBS to reduce nonspecific staining, and then rinsed in TBS. Sections were treated for 2 hours in 50% Formamide/2x SSC (0.3 M NaCl, 0.03 M sodium citrate) at 65°C, rinsed in 2x SSC for 10 min, incubated in 2 N HCl for 30 min at 37°C, rinsed in 0.1 M boric acid (pH 8.5) for 15 min, and rinsed again in TBS. Sections were incubated in 0.1% Triton X-100 (TTX; Sigma) and 3% normal horse serum (Vector Laboratories, Burlingame, CA) in TBS for 30 min, and then incubated with the primary antibody (monoclonal mouse anti-BrdU, 1:300, Roche) for 24 hours at 4 °C. Following this, sections were rinsed with TBS and incubated with the secondary antibody (biotinylated horse anti-mouse, 1:200; Vector Laboratories) for 2 hours. Sections were then rinsed in TBS, incubated in an avidin-biotinylated peroxidase complex (ABC, Vector Laboratories) for 1 hour, rinsed again in TBS, and treated for peroxidase detection with diaminobenzidine (Vector Laboratories, nickel intensified) for 4 min. Stained sections were mounted on gelatin-coated slides, counterstained with cresyl violet, dehydrated, and coverslipped.

Sox-2 labeling was adapted from a prior study (Hattiangady and Shetty, 2008a). Free-floating sections were rinsed with phosphate buffered saline (PBS) followed by 30 min in 50% methanol, 30 min in 3% hydrogen peroxide in PBS, rinsed in PBS, then blocked for 30 min in 5% normal horse serum and 0.3% Triton-X in PBS. Sections were then incubated in the primary antibody (polyclonal goat anti-Sox-2, 1:200; Santa Cruz Biotechnology, Inc.) for 24 hours at 4 °C. Following this, sections were rinsed with PBS and incubated with the secondary antibody (biotinylated horse anti-goat, 1:200; Vector Laboratories) for 2 hours. Sections were then rinsed in PBS, incubated in an ABC complex for 1 hour, rinsed again in PBS, and treated for peroxidase detection with vector grey substrate (Vector Laboratories) for 4 min. Stained sections were mounted on gelatincoated slides, dehydrated, and coverslipped. Prior to being coverslipped, BrdU-labeled sections were first counterstained with cresyl violet.

Immunofluorescent labeling for BrdU/NeuN/GFAP, Sox-2/GFAP/S100B, and Sox-2/Ki-67

Triple immunofluorescent labeling procedures for BrdU, NeuN, and GFAP were adapted from previous reports (Kuhn et al., 1996; Nilsson et al., 1999). Free-floating sections were first rinsed in PBS, followed by 15 min in 4% paraformaldehyde in 0.1 M PB, rinsed in PBS, treated in 20% methanol and 3% hydrogen peroxide in PBS, then rinsed in PBS. Sections were then denatured according to the steps used in the peroxidase method described above, rinsed in PBS, blocked in 5% normal donkey serum (Jackson Immuno) and 1% Triton-X for 30 min, and then incubated in a primary antibody cocktail (polyclonal sheep anti-BrdU, 1:150, Abcam; monoclonal mouse anti-NeuN, 1:100, Millipore; polyclonal rabbit anti-GFAP, 1:1000, Abcam) for 72 hours at room temperature (RT). Sections were then rinsed in PBS, then incubated for 2 hours with fluorescent secondary antibodies Alexa Fluor 555 donkey anti-sheep (1:200, Invitrogen), Alexa Fluor 488 donkey anti-mouse (1:200, Invitrogen), and Cy5 donkey anti-rabbit (1:200, Jackson Immuno). Sections were then rinsed in PBS, mounted on gelatin-coated slides with Vectashield anti-fading mounting medium (Vector Labs), coverslipped, and stored in the dark at 4° C.

Immunofluorescence procedures for Sox-2/GFAP/S100β and Sox-2/Ki-67 were adapted from previous reports (Hattiangady and Shetty, 2008a; Segi-Nishida et al., 2008). Free-floating sections were rinsed in PBS, then rinsed in 4% paraformaldehyde in 0.1 M PB, rinsed in PBS, treated in 20% methanol and 3% hydrogen peroxide in PBS, then rinsed in PBS. Following this, sections were blocked in 5% normal donkey serum and 1% Triton-X for 30 min, and then incubated in a polyclonal goat anti-Sox-2 primary antibody (1:200; Santa Cruz Biotechnology) for 48 hours at RT. Sections were then rinsed in PBS, then incubated for 2 hours in a fluorescent secondary Alexa Fluor 488 donkey anti-goat (1:200, Invitrogen), then rinsed in PBS. Following this, sections were then incubated in a second primary antibody cocktail (monoclonal mouse anti-GFAP, 1:1000; Millipore; polyclonal rabbit anti-S100β, 1:250; Millipore) for 36 hours at RT or in a polyclonal rabbit anti-Ki-67 (1:100; Abcam) overnight at RT. Sections were then rinsed in PBS, incubated for 2 hours with fluorescent secondary antibodies Alexa Fluor 555 donkey antimouse (1:200, Invitrogen) and Cy5 donkey anti-rabbit (1:200, Jackson Immuno), or with Alexa Fluor 555 donkey anti-rabbit (1:200, Invitrogen). Sections were then rinsed in PBS, mounted on gelatin-coated slides with Vectashield anti-fading mounting medium, coverslipped, and stored in the dark at 4° C.

Quantification of BrdU+ and Sox-2+ cells using unbiased stereology

BrdU+ and Sox-2+ cells in the dentate gyrus were counted using a modified optical fractionator method (West, 1993; West, 1999; Mouton, 2002). StereoInvestigator (Microbrightfield Inc., Williston, VT) was used to sample throughout the dentate gyrus region and count numbers of labeled cells. Every fifth section through the rostral-caudal extent of the dentate gyrus was sampled in a total of 8 sections per rat. For counting BrdU+ cells, the sampling region included the subgranular zone (SGZ) and the suprapyramidal and infrapyramidal granule cell blades. Because of the sporadic labeling of BrdU+ cells, an 80 μ m \times 80 μ m counting frame was moved throughout the entire extent of each contour surrounding the dentate gyrus region. These parameters ensured that we sampled exhaustively throughout the entire dentate gyrus such that every BrdU+ cell was counted in each section for each rat. The total number of cells counted was multiplied by 5, and then by 2 (to account for both hemispheres) to generate a total estimate. For counting Sox-2+ cells, the sampling region included the SGZ of the dentate gyrus, which was designated as an approximately 2-cell thick zone between the inner rim of the GCL and the hilus. There was a high density of Sox-2 labeling, so the optical fractionator was used to systematically sample throughout the SGZ of all rats, using an $80 \ \mu\text{m} \times 80 \ \mu\text{m}$ counting frame and $150 \ \mu\text{m} \times 150 \ \mu\text{m}$ sampling grid. Optical fractionator estimates were multiplied by 2 to account for both hemispheres. Gundersen coefficient of errors were ≤ 0.07 for all optical fractionator estimates, with a range of 0.03 to 0.07. For analysis of immunolabeled cells, an optical dissector height of 20 µm with a 2-µm guard zone was set to avoid over-sampling and stained cells were counted in each frame using a 40x objective lens. Finally, estimates of the volume of the region of dentate gyrus that was sampled for BrdU and DCX estimates were made using Cavalleri's principle (Mouton, 2002). For each section examined, the area of the dentate gyrus and SGZ was calculated by the StereoInvestigator software and was based on the boundaries of the contour tracings. Volume estimates were obtained by multiplying the section area estimates with the spacing between sampled areas. Spacing was derived by multiplying the measured, post-histology thickness of each sample by the number of sections examined, which was constant for all sections for all rats (~50% shrinkage).

Quantification of BrdU and Ki-67 double-labeled cells and Sox-2 double- and triplelabeled cells using confocal microscopy

Confocal analysis procedures were adapted from previous reports (Mirescu et al., 2006; Hattiangady and Shetty, 2008a; Segi-Nishida et al., 2008). At least 25 BrdU+ cells in the dentate gyrus and at least 100 Sox-2+ cells in the SGZ of SUP, CON, and DEF Home Cage rats (n = 4 per group) were analyzed using a Zeiss Axio Observer inverted confocal laser-scanning microscope equipped with LSM 510 software. Rat brains selected for confocal analysis had comparable Diet group means to that of the overall Diet group means for BrdU+ and Sox-2+ cell counts. Using z-sectioning at 0.5 µm intervals at 40x, BrdU+ cells were individually examined for the co-expression of NeuN or GFAP, and Sox-2+ cells were individually examined for the co-expression of GFAP and/or S100 β (\geq 100 Sox-2+ cells per rat). In a separate analysis, due to sporadic labeling of Ki-67+ cells throughout the SGZ-GCL, Sox-2+ cells in the SGZ were examined in 4 representative sections (between ~400 and 800 total Sox-2+ cells) per rat for the co-

expression of Ki-67. Lastly, at least 25 Ki-67+ cells in the SGZ-GCL (many of which were found in the SGZ, but some were also in the inner third of the GCL) were examined for the co-expression of Sox-2. Only cells that were brighter than the background were counted as immunopositive. Percentages of BrdU+ cells that co-expressed NeuN or GFAP were individually calculated for each rat analyzed and then multiplied by the total number BrdU+ cells for each rat to yield an estimated number of new neurons. Confocal microscopic analyses of Sox-2/GFAP/S100β labeled sections yielded percentages of 1) Sox-2+/GFAP+/S100β- cells, 2) Sox-2+/GFAP-/ S100β- cells, and 3) Sox-2+/GFAP+/S100β+ cells for each rat analyzed. Analyses of Sox-2/Ki-67 labeled sections yielded 1) percentages of Sox-2+ cells that co-expressed Ki-67, and 2) percentages of Ki-67+ cells that co-expressed Sox-2 for each rat analyzed.

ELISA for BDNF and NGF

For ELISA assays, all dissected samples of Home Cage rats were first weighed individually to get their wet weights. Whole tissue extracts were prepared by adding lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet NP-40, 10% glycerol, 2 mM 4-(2-aminoethyl)- benzenesulfonyl fluoride, 1 µg/ml leupeptin, 2 µg/ml aprotinin, 2 µg/ml pepstatin), followed by gentle sonication, incubation on ice for 15 min, and a brief centrifugation to clear. The supernatant from each sample was diluted 5 times with Dulbecco's PBS and acidified to pH 2.6. After 15 min of incubation at room temperature, the diluted supernatants were neutralized to pH 7.6, aliquoted and frozen for subsequent measurement of BDNF or NGF. The above procedure was performed for all samples because a previous study indicated that acidification and subsequent neutralization with base increase the amount of detectable neurotrophins in extracts of CNS tissues (Okragly and Haak-Frendscho, 1997).

The ChemiKine TM BDNF sandwich ELISA kit (Chemicon Int., Inc.) was used to assay the BDNF levels in hippocampal lysates. BDNF levels were measured according to manufacturer's instructions. First, sample/standard diluent was added to each well of the microplate (100 μ L/well). The standards were serially diluted (1:2) from 500-0 pg/mL. Standards and samples (100 μ L/well) were added to wells in duplicate and were incubated overnight at 4°C. The wells were rinsed with wash buffer and then incubated with diluted biotinylated mouse anti-BDNF monoclonal antibody (1:1000) for 2 hours at room temperature. After rinsing the plate again with wash buffer, diluted streptavidin-HRP conjugate solution (1:1000) was added to wells and incubated for 1 hour at room temperature. Plates were again washed and then warm TMB buffer was added. After 15 min incubation at room temperature, stop solution was added.

The Emax® immunoassay systems (Promega) were used to measure NGF in the samples. Flat-bottom 96 well plates (NUNC) were first coated with solution containing a polyclonal antibody against either NGF (the first primary antibody solution) prepared in carbonate coating buffer (100 μ L/well, 1:1000 dilution) and incubated for 16 hour at 4°C. Following a wash in TBST (Tris-buffered saline solution containing Tween 20), the coated wells were incubated with block and sample buffer (1X) for 1 hour at room temperature and washed again with TBST. Standard control samples for NGF were diluted serially (1:2) from 250–0 pg/mL, respectively, and plated in duplicate (100 μ L/well). The frozen ELISA samples (described above) were thawed on ice, and every
sample plated in duplicate for measurement of NGF. Following a 6 hour incubation at room temperature, wells were washed in TBST. Diluted monoclonal antibody against either NGF (the second primary antibody solution; 1:4000) was added to each well and incubated overnight at 4°C. The wells were washed in TBST, incubated with appropriate secondary antibody conjugated to peroxidase for 2.5 hours, washed again in TBST, and treated with tetramethyl benzidine (TMB) substrate for 10 min. The chromogen reaction was stopped by adding 100 μ L of 1N hydrochloric acid.

The optical density of each well was measured using the Victor³ microplate reader (PerkinElmer Life Sciences). The intensity of color was measured at a wavelength of 450 nm for all ELISAs. In order to correct for optical imperfections in the plate, readings at 540 nm were subtracted from readings at 450 nm. The standard curve was used to assess the validity of the protocol and to determine the relative concentrations of the growth factors. Values in all samples were normalized per gram of tissue assayed, and the average value for each sample was calculated separately before determining the group means.

Data analysis

Data were analyzed using ANOVAs, Fisher's post-hoc tests, and *a priori* comparisons to evaluate differences between specific group means where appropriate. A significance level of ≤ 0.05 was set for all statistical tests. Values are reported in the text as means \pm standard error of the mean (SEM). For analyses of water maze performance during delay testing, the latency and pathlength to locate the hidden platform after a 5 min or 90 min retention delay across the 3 days of delay testing was calculated for each

rat. Because each rat was only allowed a single trial to locate a novel platform after a varied retention delay, there is variability in test trial performance (e.g., performance also reflects instances when a rat accidentally bumps into the platform or when a rat swims close to the platform, but fails to precisely locate the platform). Thus, a median score for each delay (5 min vs. 90 min) was used for statistical analyses. Numbers of BrdU+ cells estimated by the optical fractionator in SUP, CON, and DEF rats were analyzed separately for rats sacrificed 24 hours versus 7 weeks after the last BrdU injection. Group mean numbers of BrdU+ cells for SUP, CON, and DEF rats sacrificed 24 hours after BrdU were used to estimate the proportion of cell loss observed 7 weeks after BrdU administration in each Home Cage and Experience SUP, CON, and DEF rat. Numbers of Sox-2+ cells estimated by the optical fractionator and were analyzed in Home Cage and Experience rats. Because daily exploration (Experience) did not alter any behavioral or neurogenesis measure, percentages of BrdU+ cells that co-expressed NeuN and GFAP, percentages of Sox-2+ cells that co-expressed GFAP, S100β, and Ki-67, and hippocampal BDNF and NGF protein levels (expressed as percent of control levels) were analyzed only in Home Cage rats. Note that subjects in each experimental condition were randomly selected from different litters (n of 1/litter). Thus, the necessary precautions have been taken to ensure that the findings are not contaminated by a lack of within litter variability.

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Results

Spatial exploration behavior

All Experience rats' behavior in the spatial exploration task was recorded with a video camera during the first 3 days of the task and then once per week for the remaining 4 weeks and each rat was scored for the following behaviors: number of contacts with objects, total time spent exploring objects, and the mean time spent exploring a given object. For the first 3 days of the exploration task, separate 3 (Diet) \times 3 (Day) mixed ANOVAs revealed that all diet groups showed an overall increase in total time spent exploring objects, F(2, 54) = 9.20, p < 0.001, and in the mean time spent exploring a given object, F(2, 54) = 5.95, p < 0.01, from Day 1 to Day 3 of spatial exploration. There were no effects of Diet or Diet × Day interactions, and no effect of Day for the number of contacts made with objects from Day 1 to Day 3. Separate 3 (Diet) × 4 (Week) mixed ANOVAs on spatial exploration behavior during weeks 2 to 5 revealed that all diet groups continued to show an overall increase in total time spent exploring objects, F(3,81) = 14.04, p < 0.001, and that there was also an overall increase in the number of contacts made with objects after Week 2, F(3, 81) = 7.94, p < 0.001 (Figure 3). However, there was no effect of Diet, nor was there a Diet × Week interaction for any behavioral measure. These data suggest that SUP, CON, and DEF rats appeared to explore novel objects similarly throughout a 5-week period.

Water maze performance

Analyses of latency to locate the hidden platform are presented below. Analyses of pathlength (data not shown) were consistent with the results of the analyses of latency



Figure 3. Object Investigation During Spatial Exploration (Experience). Total time spent exploring objects (A), mean exploration time per object (B), and total number of contacts made with objects (C) during 5 weeks (days 1-3 of Week 1 and mid-week of Weeks 2-5) of the Spatial Exploration task for Experience SUP (red), CON (blue), and DEF (grey) rats.

data. During task acquisition (pretraining), rats were given a total of 10 test trials. A 3 (Diet) \times 2 (Experience) \times 10 (Trial) mixed ANOVA revealed a significant repeated measures effect of Trial, F(9, 468) = 11.17, p < 0.001, but no effects of Diet or Experience or any significant interactions. A linear contrast analysis confirmed that across treatment groups, latency to locate the hidden platform declined as test trials progressed, F(1, 52) = 33.94, p < 0.001, although there was variability from trial to trial for some groups, which was likely due to alterations in train trial procedures (see Methods; Figure 4A). On the first 3 days of pretraining, rats were allowed to swim during train trials, and there were no significant differences between treatment groups in mean latency to locate the platform across train trials (F < 1), providing evidence that prenatal diet did not alter swimming ability or willingness to locate a novel hidden platform. Figure 4B presents the mean train trial latency, test trial 1 latency, and test trial 10 latency during pretraining for each treatment group. A 3 (Diet) \times 2 (Experience) \times 3 (Trial: train, trial 1, trial 10) mixed ANOVA revealed a significant repeated measures effect, F(2, 104)= 66.53, p < 0.001, and no effect of Diet or Experience or interactions. All groups began the task with high latencies on test trial 1 that were comparable to that which was observed during the train trial, and then all groups improved and showed decreased latencies by test trial 10. Separate linear contrasts confirmed that the latency to locate the platform significantly declined from the first to the last test trial of pretraining for all groups, (all ps < 0.05; Figure 4B). Thus, all treatment groups were able to successfully learn the DMTP water maze task with a 5 min retention delay during pretraining. After extramaze cues were obscured by placing a curtain around the maze, latency to find the





hidden platform significantly increased for all groups (Figure 4C), indicating that all rats were using extramaze cues and likely their hippocampus to navigate the maze (Morris et al., 1982).

Performance across 3 days of variable delay training was then analyzed to assess spatial memory retention as cognitive demands increased. This procedure was used because as the retention delay increases on a delayed matching-to-sample task, the hippocampus becomes more important (Winocur, 1992), as do newborn dentate granule cells (Winocur et al., 2006). During delay testing, the median latency to locate a novel hidden platform after either a 5 min or 90 min retention delay across 3 days of testing was calculated for each rat and these data are presented in Figure 5. A 3 (Diet) $\times 2$ (Experience) \times 2 (Delay) ANOVA revealed a main effect of Diet, F(2, 52) = 8.59, p < 1000.01, a main effect of Delay, F(1, 52) = 39.79, p < 0.001, and a significant Diet × Delay interaction, F(2, 52) = 6.58, p < 0.01. There was no effect of Experience for any prenatal diet group. Separate analyses of performance after the 5 min versus 90 min retention delay revealed that prenatal SUP, CON, and DEF rats found the platform rapidly when the retention delay between the train and test trial was only 5 min, with no significant differences in latency between groups (F < 1; Figure 5). When a 90 min delay was imposed, however, CON and DEF rats were impaired and had significantly higher latencies to find the hidden platform than SUP rats, which was confirmed by a main effect of Diet, F(2, 58) = 10.68, p < 0.001. Separate linear contrasts revealed that while the latency to locate the hidden platform significantly delay for both CON rats, F(1, 16) =18.02, p = 0.001, and DEF rats, F(1, 18) = 30.41, p < 0.001, the latency to find the



Figure 5. Delay Testing Water Maze Performance. Home Cage (HC; solid lines) and Experience (dashed lines) SUP (red), CON (blue), and DEF (grey) rats had similar, short latencies at the 5 min retention delay. CON and DEF rats had significantly higher latencies at the 90 min delay compared to their 5 min delay performance and compared to SUP rats. In contrast, SUP rats did not show an increase in latencies from the 5 min to 90 min delay. * significantly different at p < 0.05, # significantly different from 5 min delay at p < 0.05.

platform was as short after the 90 min delay as for the 5 min delay for SUP rats, F < 1 (Figure 5). These data indicate that in contrast to CON and DEF rats, SUP rats showed no decrement in spatial memory retention when the delay between training and testing was increased to 90 min.

Prenatal choline availability modulates cell proliferation and number of newly generated neurons in the adult dentate gyrus

To examine the addition of newly dividing cells in the subgranular zone and granular cell layer (SGZ-GCL) after 4 days of BrdU administration, SUP, CON, and DEF rats were sacrificed 24 hours after the last BrdU injection and BrdU+ cells in the SGZ-GCL were visualized and quantified in each rat using unbiased stereology. Most BrdU+ cells were evident in the SGZ, indicating that majority of the cells were newly generated (Figure 6A-C). Some BrdU+ cells were also visible in the GCL, indicating that some had survived and were migrating from the SGZ (Figure 6A-C). Analyses of the number of BrdU+ cells in the SGZ-GCL revealed a main effect of Diet, F(2, 20) = 10.36, p = 0.001. Post-hoc tests revealed that SUP rats had significantly more BrdU+ cells than both CON rats and DEF rats (ps < 0.05), while CON and DEF rats had statistically similar numbers of BrdU+ cells (Figure 6D). Differences in numbers of BrdU+ cells could not be attributed to differences in SGZ-GCL volume across prenatal diet groups (F < 1). After 4 days of BrdU administration, SUP rats had ~37% more BrdU+ cells than CON rats and ~60% more BrdU+ cells than DEF rats, supporting previous work from our laboratory demonstrating that prenatal choline supplementation enhances cell proliferation in the dentate gyrus of adult rats (Glenn et al., 2007; Glenn et al., 2008b; Wong-Goodrich et



Figure 6. BrdU Immunolabeling and Quantification 24 Hours After BrdU

Administration. SUP (*A*), CON (*B*), and DEF (*C*) rats show dentate gyrus BrdU labeling mainly in the SGZ, with SUP rats showing more labeling than CON and DEF rats. (*D*) Unbiased stereological counts of BrdU+ cells confirmed that SUP rats had significantly more BrdU+ cells in the SGZ-GCL than both CON and DEF rats. Bars in photomicrographs indicate 50 μ m. Photomicrographs in top row were taken with a 10x objective and photomicrographs in the bottom row were taken with a 40x objective. GCL, granule cell layer. SGZ, subgranular zone. H, hilus. * significantly different from CON and DEF groups at *p* < 0.01.

al.,2008a).

To examine whether prenatal choline availability and/or daily exploration in a novel environment alters the long-term survival of these newly diving cells in the dentate gyrus, Home Cage and Experience SUP, CON, and DEF rats were sacrificed 7 weeks after BrdU administration. BrdU+ cells were mainly expressed in the GCL while very few BrdU+ cells were visible in the SGZ, indicating that majority of these cells had migrated from the SGZ by 7 weeks after BrdU administration (Figure 7A-C). A 3 (Diet) \times 2 (Experience) ANOVA on the number of BrdU+ cells in the SGZ-GCL revealed a main effect of Diet, F(2, 52) = 16372, p < 0.001, but no effect of Experience or Diet \times Experience interaction (Fs < 1). SUP rats had significantly more BrdU+ cells at 7 weeks after BrdU administration than CON and DEF rats (p < 0.001) while CON and DEF rats had similar numbers of BrdU+ cells (Figure 7D). Brief daily exploration in a novel spatial environment did not alter cell survival in any diet group. Differences in numbers of BrdU+ cells could not be attributed to differences in SGZ-GCL volume across prenatal diet groups (F < 1). Increased numbers of BrdU+ cells in SUP rats 7 weeks after BrdU administration was not due to differences in rate of BrdU+ cell loss, as there were no effects of Diet, Experience, or a Diet × Experience interaction on the percentage of BrdU+ cell loss from 24 hours after BrdU administration (Fs < 1; Figure 7E). All prenatal diet groups showed similar percentages of BrdU+ cells loss ($\sim 21-25\%$), indicating that SUP rats exhibited a proportional increase in the number of BrdU+ cells observed in the SGZ-GCL at 24 hours and 7 weeks after 4 days of BrdU administration.

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Figure 7. BrdU Immunolabeling and Quantification 7 Weeks After BrdU Administration. SUP (*A*), CON (*B*), and DEF (*C*) rats show dentate gyrus BrdU labeling mainly in the GCL, with SUP rats showing more labeling than CON and DEF rats. There were no apparent effects of Experience, which was confirmed by stereological counts (*D*), so only representative sections from Home Cage rats are presented. (*D*) Unbiased stereological counts of BrdU+ cells confirmed that SUP rats had significantly more BrdU+ cells in the SGZ-GCL than both CON and DEF rats, with no effect of Experience on any prenatal diet group. (*E*) All groups showed similar relative rates of estimated BrdU+ cell loss from the 24 hour time point. Bars in photomicrographs indicate 50 µm. Photomicrographs in top row were taken with a 10x objective and photomicrographs in the bottom row were taken with a 40x objective. GCL, granule cell layer. SGZ, subgranular zone. H, hilus. * significantly different from CON and DEF groups at *p* < 0.01. Taken together, these data indicate that prenatal choline supplementation enhanced the number of newly dividing cells in the SGZ-GCL and that this increase in cell proliferation led to the increased number of surviving cells in this prenatal diet treatment group observed 7 weeks after BrdU administration.

To determine whether prenatal choline availability alters the relative proportion of newly generated cells in the SGZ-GCL that differentiated into mature neurons or astrocytes, BrdU+ cells in a subgroup of Home Cage SUP, CON, and DEF rats (n = 4 per group, ≥ 25 cells per rat) were examined for the co-expression of NeuN (mature neuronal marker) or GFAP (astrocyte marker) using triple immunofluorescence and confocal analysis (Figure 8). Because daily spatial exploration had no effect on the number of BrdU+ cells in the SGZ-GCL in any prenatal diet group, Experience rats were not included in the analysis. Confocal analysis of BrdU+ cells revealed that the majority of BrdU+ cells found in the SGZ-GCL of SUP ($86.84 \pm 2.46\%$), CON ($85.61 \pm 1.49\%$), and DEF $(74.12 \pm 3.08\%)$ rats were also immunopositive for NeuN (Figure 9), which is consistent with previous reports (Cameron et al., 1993; Dayer et al., 2003). A one-way ANOVA revealed a main effect of Diet for the percentage of BrdU+/NeuN+ neurons, F(2, 9) = 8.38, p < 0.01. Both SUP and CON rats had a significantly greater proportion of BrdU+ cells that also expressed NeuN (about 11% more) than DEF rats (ps < 0.01). A very small proportion of BrdU+ cells co-expressed GFAP (SUP = $5.71 \pm 1.93\%$; CON = $3.05 \pm 0.32\%$; DEF = $5.37 \pm 0.90\%$), which is consistent with previous reports (Cameron et al., 1993), and analyses did not reveal an effect of Diet (Figure 9), indicating that prenatal choline availability did not alter the relative proportion of newly generated



Figure 8. Phenotypic Analysis of BrdU+ Cells in the adult Dentate SGZ-GCL.

Confocal images showing examples of BrdU+ cells co-labeled (arrows) with the mature neuronal marker NeuN (green), the mature astrocyte marker GFAP (blue), or neither (arrow head). Scale bars indicate 25 μ m. Confocal images taken with a 40x objective. GCL, granule cell layer. SGZ, subgranular zone. H, hilus.



Figure 9. Phenotype of BrdU+ Cells in the Adult Dentate SGZ-GCL at 7 Weeks After BrdU Administration. SUP and CON rats show similar percentages of neuronal differentiation, but DEF rats had a slightly lower percentage of BrdU+ cells that coexpressed NeuN that was concomitant with a slightly higher percentage of unclassified (Other) BrdU+ cells (n = 4 per group). There was no effect of diet on the rate of astrocytic differentiation of BrdU+ cells. * significantly different at p < 0.05.

astrocytes. Analyses on the percentage of remaining BrdU+ cells that did not express NeuN or GFAP revealed a main effect of Diet, F(2, 9) = 8.24, p < 0.01. Prenatal DEF rats had a significantly greater percentage ($20.51 \pm 3.51\%$) of these unclassified BrdU+ cells than both SUP ($7.45 \pm 1.58\%$) and CON ($11.34 \pm 1.23\%$) rats (ps < 0.05), while SUP and CON rats were not statistically different.

Percentages of BrdU+ cells that co-expressed NeuN, GFAP, or neither were used to calculate the estimated number of new neurons, astrocytes, or unclassified cell types in the SGZ-GCL of Home Cage SUP, CON, and DEF rats. In absolute terms, SUP rats had significantly more BrdU+ neurons (1673.62 ± 134.12) compared to CON ($1248.66 \pm$ 102.16) and DEF (900.59 \pm 57.96) rats (ps < 0.05). The 11% reduction in the proportion of BrdU+/NeuN+ neurons in DEF rats also translated into a significant net decrease in numbers of newborn neurons compared to CON rats (p < 0.01). Although prenatal diet did not alter the proportion of BrdU+ cells that expressed GFAP, absolute numbers of BrdU+ astrocytes were significantly different across all three diet groups with SUP rats having the highest number (104.31 \pm 13.66), followed by DEF rats (65.33 \pm 5.78), then CON rats (43.73 \pm 2.85; all ps \leq 0.01). Estimated numbers of BrdU+ cells that did not express NeuN or GFAP were significantly higher in prenatal DEF rats (247.07 ± 19.50) compared to both SUP (145.07 \pm 16.67) and CON (163.86 \pm 12.73) rats (ps < 0.01), while there was no significant difference between SUP and CON rats. Taken together, these data indicate that prenatal choline supplementation's enhancement of cell proliferation translated into a net increase in the number of surviving newborn neurons and astrocytes in the adult dentate gyrus, while prenatal choline deficiency's reduction in

the rate of neuronal differentiation led to a net decrease in the number of surviving newborn neurons in the adult dentate gyrus.

To determine whether there was a relationship between newborn dentate cell survival and performance on the DMTP task, numbers of BrdU+ cells were correlated with water maze performance. Numbers of BrdU+ cells observed 7 weeks after BrdU administration did not significantly correlate with median latency after a 5 min retention delay, but there was a moderate, but significant negative linear correlation between the number of BrdU+ cells and median latency after the 90 min delay, which is where prenatal diet differences were observed, r(58) = -0.35, $R^2 = 0.17$, p < 0.05 (Figure 10). The correlation suggested that as numbers of BrdU+ cells surviving to 7 weeks increased, performance after the long delay in the water maze improved.

Prenatal choline availability does not alter the number, but does influence the phenotype and mitotic status of putative adult stem/progenitor cells in the dentate gyrus

One mechanism of altered adult hippocampal neurogenesis by prenatal choline availability may be changes in the number, phenotype, and/or proliferative capacity of resident stem/progenitor cells in the SGZ that give rise to newborn granule cells in the adult dentate gyrus. To determine whether prenatal choline availability and/or daily exploration in a novel spatial environment alters the total number of progenitor cells in the dentate SGZ, cells immunopositive for Sox-2, a marker of neural stem cells in the SGZ of the dentate gyrus (Komitova and Eriksson, 2004; Suh et al., 2007), were visualized and quantified for each rat using unbiased stereology. Sox-2+ cells were expressed throughout the dentate gyrus, but there was a greater density of Sox-2+ cells in



Figure 10. Correlation Between Water Maze Performance and Adult Hippocampal Neurogenesis. A plot of median latency scores (s) vs. the number of BrdU+ cells in the SGZ-GCL observed at 7 weeks after BrdU administration for individual Home Cage and Experience SUP (red circles), CON (blue squares), and DEF (grey triangles) rats shows no correlation between number of BrdU+ cells and performance after the 5 min delay, $R^2 = 0.01$ (*A*), but a significant moderate negative correlation after the 90 min delay, r(58) = -0.35, $R^2 = 0.17$, p < 0.05 (*B*).

the SGZ in comparison to the GCL, molecular layer, and hilus (Figure 11), which is consistent with previous reports (Komitova and Eriksson, 2004; Brazel et al., 2005; Hattiangady and Shetty, 2008a). The density of Sox-2+ cells in the SGZ appeared to be similar across all treatment groups (Figure 11). The total number of Sox-2+ cells in the SGZ were estimated using the optical fractionator method, and estimates were subjected to a 2 × 2 ANOVA with Diet and Experience as between-subjects factors. The ANOVA revealed no effects of Diet, Experience, or Diet × Experience interaction on the total number of Sox-2+ cells in the SGZ (*F*s < 1; Figure 11). There were also no significant differences across treatment groups in SGZ volume estimates (*p*s > 0.05). Thus, neither prenatal choline availability nor brief daily spatial exploration for 5 weeks altered the total number of Sox-2+ cells in the neurogenic SGZ of the dentate gyrus.

Sox-2, however, is expressed by several cell types in the dentate gyrus (Komitova and Eriksson, 2004; Hattiangady and Shetty, 2008a). To examine whether prenatal choline availability alters the composition of putative stem cell types in the SGZ, fractions of Sox-2+ cells in the SGZ from Home Cage SUP, CON and DEF rats (n = 4 per group, ≥ 100 Sox-2+ cells per rat) were analyzed for the co-expression of GFAP (radial glia-like stem cell marker) and S100 β (mature astrocyte marker) using triple immunofluorescence and confocal analysis (Figure 12). Because daily spatial exploration did not alter the total number of Sox-2+ cells in the SGZ in any prenatal diet group, Experience rats were not included in the analysis. The majority of Sox-2+ cells in the SGZ were stem/progenitor cells and did not express the mature astrocytic marker S100 β (Figure 13), which is consistent with previous studies (Suh et al., 2007; Hattiangady and

Shetty, 2008a). Percentages of Sox-2+ cells that were GFAP+/S100β- (Type-1 radial glia-like stem cells), GFAP-/S100β- (Type-2 stem cells and other Sox-2+ progenitor cells), and GFAP+/S100β+ (mature astrocytes) were subjected to a one-way ANOVA with Diet as a between-subjects factor. For the percentage of Sox-2+ cells that were GFAP+ and S100β-, there was a main effect of Diet, F(2, 9) = 6.81, p < 0.05, where SUP rats had a significantly higher percentage (49.86 ± 0.67%) of these putative Type-1 radial glia-like stem cells than CON (42.49 ± 1.68%) and DEF (44.96 ± 1.72%) rats (ps < 0.05; Figure 13). As a result of this increase in the percentage of putative Type-1 stem cells, SUP rats did tend to show a smaller percentage of Sox-2+/GFAP-/S100β- cells (Type-2 cells and other Sox-2+ progenitors) compared to CON rats (Figure 11B); however, the main effect of Diet did not reach statistical significance (p = 0.12). There was no effect of Diet for the percentage of Sox-2+ cells that were GFAP+ and S100β+ (F < 1; Figure 13), suggesting no effect of prenatal choline availability on Sox-2+ expression in mature astrocytes.

To determine whether prenatal choline availability alters the proliferative status of neural stem/progenitor cells in the adult dentate gyrus, Sox-2+ cells co-expressing the proliferative marker Ki-67 in the SGZ were visualized using dual immunofluorescence and confocal microscopy in the same subgroup of SUP, CON, and DEF rats (n = 4 per group). The Ki-67 protein is an endogenous cellular marker strictly associated with cell proliferation and is present in all active phases of cell division (Scholzen and Gerdes, 2000). The percentages of Sox-2+ cells that co-expressed Ki-67 that were generated in the current experiment were considerably smaller than that of a previous study using





Figure 11. Sox-2 Immunolabeling and Quantification in the Adult Dentate SGZ.

SUP (*A*), CON (*B*), and DEF (*C*) rats show Sox-2 throughout the dentate gyrus labeling, with a greater density of Sox-2+ cells in the SGZ than the GCL and ML and no apparent differences between diet groups. There were no apparent effects of Experience, which was confirmed by stereological counts (*D*), so only representative sections from Home Cage rats are presented. (*D*) Unbiased stereological counts of Sox-2+ cells confirmed no effect of Prenatal Diet or Experience on the number of Sox-2+ cells in the SGZ. Bars in photomicrographs indicate 50 μ m. Photomicrographs in top row were taken with a 10x objective and photomicrographs in the bottom row were taken with a 40x objective. GCL, granule cell layer. SGZ, subgranular zone. H, hilus. ML, molecular layer.



Figure 12. Phenotypic Analysis of Stem/Progenitor Cells in the Adult Dentate SGZ. Staining for Sox-2 (green) was used as a marker for all stem/progenitor cells. Staining for GFAP (red) was used as a marker for radial glial-like Type-1 cells. Staining for S100 β (blue) was used as a marker for mature astroctyes. Confocal micrographs show Sox-2-labeled GFAP+ but S100 β -negative cells (Type-1 cells; arrows), Sox-2-labeled but GFAP- and S100 β -negative cells (Type-2 cells and other progenitors; arrowheads), and Sox-2-labeled GFAP+ and S100 β + cells (astrocytes; indented arrowheads). Scale bars indicate 25 μ m. Confocal images were taken with a 40x objective. GCL, granule cell layer. SGZ, subgranular zone. H, hilus.



Figure 13. Quantification of Phenotype of Sox-2+ Cells in the Adult Dentate SGZ. SUP rats had a significantly higher percentage of Type-1 radial glia-like Sox-2+ cells (GFAP+/S100 β -) than CON and DEF rats. Prenatal diet did not significantly affect the proportion Sox-2+ Type-2 cells or astrocytes in the SGZ. * significantly different at p < 0.05.

young adult (4 mo) male Fischer rats (Hattiangady & Shetty, 2008). However, due to the sporadic labeling of Ki-67 throughout the SGZ, the current experiment examined a much larger sample of Sox-2+ cells (400-800 total Sox-2+ cells) than the previous study (100 total Sox-2+ cells). Differences in sampling methods, as well as differences due to rat strain, age, and sex may have contributed to these disparate findings.

There were no significant differences between diet groups in the total number of Sox-2+ cells analyzed for co-expression of Ki-67 (F = n.s.), consistent with the Sox-2+ cell count data generated using the optical fractionator. Compared to the large number of Sox-2+ cells present throughout the SGZ, there was sporadic labeling of Ki-67+ cells with some Ki-67+ cells in the process of migrating toward the GCL (Figure 14), which is consistent with previous studies (Rao et al., 2006; Hattiangady and Shetty, 2008a). Most Ki-67+ cells, however, were found in the SGZ and often appeared in pairs and sometimes in clusters, and a vast majority of Ki-67+ cells in the SGZ co-expressed Sox-2 (Figure 14). Similar to the BrdU results, SUP rats also tended to show a greater density of Ki-67 labeling throughout each of the sections analyzed than CON and DEF rats (Figure 14). This was supported by a one-way ANOVA on the percentage of Sox-2+ cells that were also Ki-67+, which revealed a main effect of Diet, F(2, 9) = 5.54, p < 0.05, where SUP rats had a significantly higher percentage $(8.84 \pm 1.35\%)$ of Sox-2+ cells in the SGZ that co-expressed Ki-67 than both CON (4.63 \pm 0.26%) and DEF (4.59 \pm 1.15%) rats (ps < 0.05; Figure 15A). There was no significant difference between CON and DEF rats. Consistent with the BrdU findings, these data indicate that SUP rats exhibit higher levels of proliferation by neural stem/progenitor cells in the adult dentate gyrus.



Figure 14. Dual Immunofluorescence Analysis of Mitotic Activity of Sox-2+ Stem/ Progenitor Cells. SUP (*A*), CON (*B*), and DEF (*C*) rats show Ki-67 immunofluorescence labeling in the dentate gyrus mainly in the SGZ with a few Ki-67+ cells in the GCL (Ki-67+ cells denoted by arrows), and SUP rats tended to show more labeling than CON and DEF rats. (*D*) Confocal images showing Sox-2+ cells in the SGZ co-labeled with Ki-67 (arrows) or not co-labeled (arrowheads). Photomicrograph images in *A*-*C* were taken with an epifluorescence microscope with a 10x objective. Confocal images taken with a 40x objective. Scale bars indicate 50 µm in *A*-*C* and 25 µm in *D*. GCL, granule cell layer. SGZ, subgranular zone. H, hilus.



Figure 15. Mitotic Status of Stem/Progenitor Cells in the SGZ and Phenotypic Makeup of Dividing Cells in the SGZ-GCL of the Adult Dentate Gyrus.

(*A*) SUP rats had a significantly higher percentage of Sox-2+ cells in the SGZ that coexpressed Ki-67 than both CON and DEF rats (n = 4 per group). (*B*) There was no effect of prenatal diet on the percentage of Ki-67+ cells in the SGZ-GCL of the dentate gyrus region that co-expressed Sox-2 (n = 4 per group). * significantly different from CON and DEF rats at p < 0.05.

In the sections labeled for Sox-2 and Ki-67, not all observed Ki-67+ cells were restricted to the SGZ, and a number of Ki-67+ cells did not express Sox-2. As Ki-67+ cells started to migrate beyond the parameter used to define the SGZ (see Methods), Sox-2 co-expression became less frequent. Doublecortin-positive neuroblasts also undergo an initial period of cell division and may constitute this Sox-2-negative population of dividing cells (Kronenberg et al., 2003; Rao and Shetty, 2004). To assess whether prenatal choline availability influences the phenotypic characteristics of dividing cells in the adult dentate gyrus, numbers of Ki-67+ cells throughout the dentate gyrus (SGZ and GCL) were examined for the co-expression of Sox-2 in the same labeled tissue sections of SUP, CON, and DEF rats (n = 4 per group; ≥ 25 cells per rat). The majority of the Ki-67+ cells in the SGZ-GCL in SUP (87.15 ± 4.79), CON (85.70 ± 2.42), and DEF (76.38 ± 100) 7.69) rats were indeed Sox-2+. Analyses revealed no significant effect of Diet on the percentage of Ki-67+/Sox-2+ cells in the SGZ-GCL (Figure 15B), suggesting that prenatal choline status does not influence the composition of proliferating Sox-2+ versus Sox-2-negative cell types within the SGZ-GCL of the adult dentate gyrus.

Prenatal choline availability alters expression of BDNF and NGF in adult hippocampus Increased neurotrophic/growth factor expression in the hippocampus can promote adult hippocampal neurogenesis (Lee et al., 2002b; Frielingsdorf et al., 2007) and may be one molecular mechanism underlying enhancements in hippocampal neurogenesis in adult SUP rats. Hippocampal protein levels of BDNF and NGF were quantified in Home Cage SUP, CON, and DEF rats and were expressed as percent of control values. A one-way ANOVA revealed a significant main effect of Diet for both BDNF, F(2, 27) = 3.38, p = 0.05, and NGF, F(2, 28) = 8.54, p = 0.001. As predicted, a planned comparison revealed that SUP rats had significantly higher levels of hippocampal BDNF than CON rats (p =0.03, one-tailed; Figure 16), which is consistent with previous findings in young adult female rats (Glenn et al., 2007). Surprisingly, however, DEF rats also had significantly higher levels of BDNF than CON rats (p < 0.05), but were not significantly different from SUP rats (Figure 16). For hippocampal NGF, SUP rats had significantly higher levels of NGF than both CON and DEF rats (ps < 0.01), with no significant difference between CON and DEF rats. These data extend prior work showing enhanced hippocampal NGF in 20 day-old and 3 month-old male rats that were prenatally supplemented with choline (Sandstrom et al., 2002), and demonstrate that this effect also occurs in females and extends further into adulthood.

Discussion

Experiment 1 demonstrated that rat offspring from dams that were supplemented with choline on ED 12-17 compared to offspring of control-fed dams showed enhanced spatial memory retention on a DMTP water maze task, which adds to previous studies reporting that prenatal choline supplementation enhances memory precision and capacity in adulthood (Meck et al., 1988; Meck et al., 1989; Williams et al., 1998; Meck and Williams, 2003; Meck et al., 2008; Wong-Goodrich et al., 2008a). In the current experiment, spatial memory performance at the long, but not short delay was significantly correlated with newborn cell survival in the adult dentate gyrus, suggesting a relationship between memory retention and adult hippocampal neurogenesis as well as further highlighting adult hippocampal neurogenesis as one potential mechanism for prenatal



Figure 16. Adult Hippocampal Neurotrophic Factor Expression. Both SUP and DEF rats had significantly more hippocampal BDNF protein than CON rats with no difference between SUP and DEF rats. Similarly, SUP rats had significantly higher hippocampal NGF protein content than both CON and DEF rats, but there was no difference between CON and DEF rats. * significantly different at p < 0.05. All assays were conducted on Home Cage rats.

choline's memory enhancing effects. Experiment 1 also revealed what potential factors may underlie the enhancement in adult hippocampal neurogenesis in prenatally choline supplemented rats, including an increase in the number of newborn cells in the dentate gyrus, an increase in number of newborn neurons at 7 weeks after cell birth, an increase in the proportion of Type-1 radial glia-like stem cells in the dentate SGZ, and an increase in the mitotic rate of neural stem/progenitor cells in the dentate SGZ. Although rat offspring of dams that were fed a choline deficient diet performed similarly to control-fed offspring in the DMTP water maze task, prenatally choline deficient rats had reduced numbers of surviving newborn neurons due to slightly reduced rates of neuronal differentiation of newborn cells at 7 weeks after cell birth, despite having comparable levels of cell division to that of control-fed rats.

Importantly, Experiment 1 confirmed previous findings that prenatal choline supplementation enhances the number of newly dividing cells and doublecortin-positive immature neurons in the adult dentate gyrus (Glenn et al., 2007; Wong-Goodrich et al., 2008a), and extended this work to show that this supplementation-induced increase in adult dentate cell proliferation translated into a net increase in the number of surviving newborn granule cells that were present at least 7 weeks after cell division and expressed markers of neuronal maturation. In the current experiment, prenatal choline supplementation both enhanced cell division *and* increased numbers of those newly born cells weeks after cell division without altering the rate of BrdU+ cell loss, suggesting that prenatal choline supplementation enhances both the proliferation and maintains the *survival* of a larger number of newborn neurons in the adult dentate gyrus. Enhanced

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proliferation without alterations in relative rates of apoptosis has also been demonstrated in the fetal hippocampus on ED 17-20 taken from fetuses in pregnant dams that were supplemented with choline on ED12-17 (Albright et al., 1999; Craciunescu et al., 2003), suggesting that this enhancement in neurogenesis initiated during early development by dietary choline supplementation may initiate organizational changes in the brain that programs the capacity for enhanced neurogenesis throughout adulthood.

The current experiment investigated potential mechanisms for prenatal choline's modulation of adult hippocampal neurogenesis. Perhaps somewhat surprising was that prenatal choline availability did not alter the overall number of putative Sox-2+ stem/progenitor cells in the adult SGZ. This may have been partially due to the fact that Sox-2 is expressed by many cell types within the adult SGZ, including mature astrocytes and oligodendrocyte progenitor cells in addition to neural stem/progenitor cells (Komitova and Eriksson, 2004; Steiner et al., 2004; Hattiangady and Shetty, 2008a). Experiment 1, therefore, examined the phenotypic features of these stem/progenitor cells and revealed that enhanced adult hippocampal neurogenesis in SUP rats may be at least in part due to an increase in the proportion of Type-1 radial glia-like stem cells and an increase in the mitotic activity of stem/progenitor cells in the SGZ. Indeed, because prenatal choline supplementation did not alter rates of BrdU+ cell loss or neuronal differentiation, this suggests that increased neurogenesis in SUP rats in the current experiment is most likely primarily driven by the activity of neural stem/progenitor cells.

During development, radial glial cells provide the necessary scaffold for the genesis of the dentate gyrus and other regions of hippocampus (Stanfield and Cowan,

1979; Forster et al., 2002; Imura et al., 2003; Anthony et al., 2004), and prior work has indicated that prenatal choline supplementation enhances neurogenesis in the fetal hippocampus (Albright et al., 1999; Craciunescu et al., 2003). Taken together with the data from the current experiment, these findings suggest that increased prenatal choline availability may program the developing dentate gyrus to have a larger pool of radial glial-like stem cells throughout life. Though the mitotic activity of Type-1 radial glia-like cells is limited (Kronenberg et al., 2003), it is possible that a small increase in the pool of radial glia-like stem cells, which provide a principal source (i.e., are able to divide and give rise to amplifying daughter cells) of neurogenesis in the adult hippocampus (Seri et al., 2001; Garcia et al., 2004; Komitova and Eriksson, 2004; Steiner et al., 2006; Liu et al., 2010), would effectively confer a measurable increase in the number of newly generated neurons. Previous reports have also shown that this population of adult neural stem cells can be modulated in adulthood (e.g., seizures, NMDA receptor antagonists, aging), and that changes in the number of radial glia-like stem cells can result in appreciable altered numbers of newborn neurons (Nacher et al., 2003; Segi-Nishida et al., 2008; Steiner et al., 2008, but see Hattiangady and Shetty, 2008a).

In addition to slightly increasing the proportion of Type-1 radial glia-like stem cells, Experiment 1 also showed that prenatal choline supplementation increased the mitotic activity of Sox-2+ neural stem/progenitor cells in the adult SGZ. These findings parallel the current findings that show increased numbers of BrdU+ cells in SUP rats after 4 days of BrdU administration, as well as with our previous BrdU findings showing lifelong enhancements in adult dentate cell proliferation with prenatal choline supplementation (Glenn et al., 2007; Glenn et al., 2008b; Wong-Goodrich et al., 2008a). Although SUP rats showed a greater proportion of proliferating Sox-2+ stem/progenitor cells compared to control-fed rats and DEF rats, the total number of Sox-2+ cells as well as the proportion of Sox-2+ putative Type-2 (non-radial glia-like) cells, which have been shown to be highly proliferative (Kronenberg et al., 2003), was not altered by prenatal choline availability. These seemingly contradictory data can be reconciled because the asymmetric division of Sox-2+ stem/progenitor cells, on a population level, is part of an internal mechanism used to maintain a stable pool of Sox-2+ stem/progenitor cells in the SGZ, and it is likely that the increase in neurogenesis in SUP rats is associated with an increase in the generation of Sox-2+ cell-derived daughter cells. In a recent study, Suh and colleagues (2007) demonstrated that adult mice that ran on running wheels had increased cell division of Sox-2+ stem cells in the adult SGZ compared to non-runners, but the total number of Sox-2+ stem cells did not change. What did change, however, was that running dramatically increased the number of newly born DCX+ neuronal precursors. Thus, running-induced proliferation of Sox-2+ stem cells in the adult SGZ does not lead to an overall increase in the total population of Sox-2+ cells, but is associated with the generation of more DCX+ precursors, which contributes to runninginduced neurogenesis. Indeed, prenatal choline supplemented rats also show increased numbers of DCX+ cells in the adult hippocampus (Glenn et al., 2007), which, taken together with the findings from the current experiment, further supports this model. Due to constraints imposed by the tissue post-fixing and immunolabeling procedures employed by the current experiment, whether prenatal choline supplementation's

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enhancement of SGZ stem/progenitor cell mitotic activity was due to increased proliferation of Type-1 cells or Type-2 cells or both could not be deciphered. Because there is evidence that proliferation of both types of stem cells can be modulated in adulthood (Kronenberg et al., 2003; Suh et al., 2007; Segi-Nishida et al., 2008), and given that SUP rats appear to show a slightly greater proportion of Type-1 radial gliallike stem cells in the SGZ, this question is of interest for future studies.

Whereas the proliferative effects of running on neural precursors in the adult dentate gyrus are transient (Kronenberg et al., 2006), prenatal choline supplementation's enhancement of cell division in the dentate gyrus remarkably persists throughout adulthood (Glenn et al., 2007; Wong-Goodrich et al., 2008a), and even into old age (Glenn et al., 2008b). A recent study found a similar pattern of findings in male Fischer rats where numbers of Sox-2+ cells in the SGZ did not change with age, but the proliferative capacity of these cells was altered with age, dramatically declining from young adulthood (4 mo) to middle-age (12 mo) to aging (24 mo) (Hattiangady and Shetty, 2008a). Although this study did not find that aging altered the phenotype of Sox-2+ stem/progenitor cells in the adult SGZ (Hattiangady and Shetty, 2008a), a previous study revealed age-related declines in radial glia-like stem cells in the adult dentate gyrus in adult female rats (Nacher et al., 2003). In light of the current data, one hypothesis is that prenatal choline supplementation confers lifelong increases in both number of radial glia-like stem cells as well as the mitotic activity of stem/progenitor cells in the SGZ, as both of these factors appear to be vulnerable to aging, to support lifelong enhancements in hippocampal neurogenesis into old age (Glenn et al., 2008b).

SUP rats have more newborn cells in the adult dentate gyrus and these additional cells are surviving at the same rate as control-fed rats (i.e., no increase in the rate of BrdU+ cell loss). These data suggest that there may be an increased demand for hippocampal trophic support in prenatally choline supplemented rats. Indeed, this was revealed by elevated expression levels of hippocampal BDNF and NGF in SUP rats, which is consistent with previous reports (Sandstrom et al., 2002; Glenn et al., 2007). Importantly, both BDNF and NGF promote the survival of newborn neurons in the adult hippocampus (Lee et al., 2002a; Sairanen et al., 2005; Frielingsdorf et al., 2007; Donovan et al., 2008). Thus, with an increase in the mitosis of newborn dentate cells, a prenatally choline supplemented brain would optimally benefit from enhanced hippocampal trophic support (i.e., BDNF, NGF, etc.) in order to support the survival of those additional dentate granule cells.

Alternately, it is also possible that prenatal choline's effects on adult hippocampal growth factor expression inherently produces changes in adult hippocampal neurogenesis and other features of adult hippocampal plasticity (e.g., LTP, synaptic plasticity, dendritic spine density) that support cognitive function (Pyapali et al., 1998; Jones et al., 1999; Montoya and Swartzwelder, 2000; Meck et al., 2008). Other factors contributing to the adult hippocampal microenvironment that are known to regulate properties of adult hippocampal neurogenesis may also be altered by prenatal choline status, such as the vascular niche of the adult SGZ (Palmer et al., 2000; van Praag et al., 2005; Hattiangady and Shetty, 2008a) and hippocampal neuroinflammation (Monje et al., 2003; Das and Basu, 2008). Although prenatally choline supplemented rats show increased hippocampal

VEGF protein and reduced levels of markers of hippocampal neuroinflammation in old age (Glenn et al., 2008a; Glenn et al., 2008b), future studies are needed to determine whether an altered vascular niche *per se* in the SGZ and/or hippocampal neuroinflammation contributes to prenatal choline's lifelong effects on hippocampal neurogenesis throughout adulthood.

Enhanced neurogenesis might be one mechanism for prenatal choline supplementation's enhancement of spatial memory ability. In the current experiment, a DMTP water maze task was used to assess spatial memory. Varying the retention delay on matching-to-sample-like tasks is useful for examining the effects of cognitive load on hippocampal memory function because as delays get longer, the hippocampus becomes more important (Winocur, 1992), as do newborn dentate granule cells (Winocur et al., 2006). Indeed, inhibiting neurogenesis via cranial irradiation produces deficits on a nonmatching-to-sample task after a longer, but not shorter retention delay (Winocur et al., 2006). Other studies using reference memory tasks have also highlighted a role for hippocampal neurogenesis after longer retention delays (Snyder et al., 2005; Deng et al., 2009). Consistent with these findings, Experiment 1 revealed that compared to controlfed and DEF rats, SUP rats showed an enhancement of spatial memory performance only after the long, but not short retention delay in the DMTP water maze task. There was also a significant moderate overall correlation between the number of surviving BrdU+ cells (at least 7 weeks after cell division) and water maze performance after the long, but not short retention delay, offering some support for a role for newborn neurons under conditions when cognitive load (i.e., retention delay) is increased. In contrast to spatial

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memory performance, all prenatal diet groups showed similar rates of learning during training. Consistent with these findings, a number of studies using various ablation approaches have directly implicated adult hippocampal neurogenesis in memory retention, while excluding its involvement in spatial learning (Madsen et al., 2003; Raber et al., 2004b; Snyder et al., 2005; Saxe et al., 2006; Winocur et al., 2006; Deng et al., 2009; Kitamura et al., 2009).

Experiment 1 revealed clear, robust effects of prenatal choline supplementation on spatial memory and hippocampal neurogenesis, but was less sensitive at capturing potential effects of prenatal choline deficiency on brain and behavior. Prenatal choline deficiency had no effect on spatial memory performance in the DMTP water maze task: DEF rats performed similarly to control-fed rats after both the short and long retention delay. One explanation for these findings is that the shorter retention delay did not impose a great enough challenge for deficient rats to reveal a cognitive deficit, while the longer retention delay presented a significant challenge for both control-fed and DEF rats. Indeed, prior work has demonstrated that only when the cognitive demands of the task are sufficiently increased, will impairments in task performance (compared to control-fed rats) emerge in prenatally choline deficient rats (Meck and Williams, 1997c; Meck and Williams, 1999).

Contrary to what was predicted, Experiment 1 detected a slight decrease in the rate of neuronal differentiation in DEF rats compared to control-fed rats, which translated into a net decrease in the number of newborn neurons in the dentate gyrus. Surprisingly, DEF rats also had higher hippocampal BDNF protein than control-fed rats. These last two

findings are in contrast to the prior study that reported no effect of prenatal choline deficiency on the number of DCX+ cells in the dentate gyrus or on hippocampal BDNF expression (Glenn et al., 2007), but the prior study did not evaluate long-term survival of newborn granule cells or co-label for markers of neuronal differentiation or maturation, and hippocampal BDNF expression was evaluated in a group of naïve rats that were not exposed to any behavioral tasks (e.g., extensive water maze training, as in the current experiment). Regardless of an observed increase in hippocampal BDNF in the current experiment, however, DEF rats in the current experiment did not exhibit enhanced neurogenesis and even showed slightly reduced neuronal differentiation. Prenatal choline deficiency also had no effect on the number of newly divided cells in the dentate gyrus, consistent with the prior study (Glenn et al., 2007), nor on any stem/progenitor cell measure evaluated, suggesting that prenatal choline deficiency does not affect basal rates of proliferation *per se* in the adult dentate gyrus.

Little is known about what factors may mediate neuronal differentiation of adultborn cells in the dentate gyrus, but NT-3 signaling may be one contributing factor. Similar to the current experiment, reduced neuronal differentiation, rather than proliferation, of newborn neurons (but not glia) has also been demonstrated in mutant mice in which the gene for NT-3 is deleted (Shimazu et al., 2006). Prenatally choline deficient rats may have lower hippocampal expression levels of NT-3 or of high-affinity receptors for NT-3 (TrkB and TrkC). Impaired TrkB signaling may also partially explain a lack of effect of prenatal choline deficiency on adult dentate cell proliferation despite increased hippocampal BDNF in the current experiment. It is also possible that the

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reduced proportion of BrdU+/NeuN+ neurons observed in DEF rats could also be due to delayed maturation of newborn granule cells, as opposed to reductions in neuronal differentiation *per se*. Future studies are needed to elucidate this possibility, as well as the functional implications for decreased neuronal differentiation/maturation of newly born cells in the dentate gyrus of prenatally choline deficient animals, and whether this decrease persists into late adulthood when perturbed neuronal differentiation and maturation may be further exacerbated by age (Rao et al., 2005; van Praag et al., 2005; Nyffeler et al., 2008).

Interestingly, Experiment 1 demonstrated that two opposing dietary manipulations of the same nutrient, choline, during early development did not produce a dosedependent-like effect on adult hippocampal neurogenesis. Rather, prenatal choline supplementation and deficiency appeared to exert their effects on different features of hippocampal neurogenesis in the adult brain with different degrees of magnitude, a finding that is consistent with the previous study showing differential effects of prenatal choline status on basal versus reactive neurogenesis (Glenn et al., 2007).

Based on prior work (Glenn et al., 2007), one feature that was initially predicted to be altered by prenatal choline availability in the current experiment was properties of reactive neurogenesis in response an enriching experience. A goal of Experiment 1 was also to determine whether or not enrichment-induced neurogenesis in control-fed rats would improve memory to that of prenatally supplemented rats, or whether prenatal choline supplementation offers any additional cognitive benefit above enrichment. Although the previous study from our lab revealed that goal-directed spatial exploration on a radial arm maze for 3 weeks increased numbers of BrdU+ and DCX+ cells in the adult dentate gyrus in control-fed rats, but not in prenatally choline deficient rats (Glenn et al., 2007), the behavioral manipulation in the current experiment was not sufficient to alter any neurogenesis measure or behavioral measure in any prenatal diet group. Perhaps the animals did not receive enough enrichment, the effects waned with time, or the additional experience of water maze training, with both an exercise and a learning component (Gould et al., 1999a; van Praag, 2008), may have equally stimulated neurogenesis in both Home Cage and Experience groups to comparable levels. Although the current experiment was likely not sensitive enough to capture potential effects of prenatal choline deficiency on reactive neurogenesis in the intact adult hippocampus, future studies that employ a more reliable manipulation, such as enriched housing conditions (Kempermann et al., 1997b; Kempermann et al., 1998; Nilsson et al., 1999), are needed.

EXPERIMENT 2: PRENATAL CHOLINE AVAILABILITY MODULATES OLFACTORY BULB NEUROGENESIS

Prenatal choline supplementation during embryonic days (ED) 12-17 reliably increases basal levels of adult hippocampal neurogenesis, primarily through enhancing cell proliferation (Experiment 1; Glenn et al., 2007; Glenn et al., 2008b; Wong-Goodrich et al., 2008a). In contrast, prenatal choline deficiency during ED 12-17 does not appear to dramatically alter the basal proliferation rate of newborn neurons in the adult hippocampus (Experiment 1; Glenn et al., 2007), but may slightly reduce the proportion of newborn cells that eventually express markers of neuronal maturity, which may translate into a net decrease in the population of newborn dentate neurons (Experiment 1). While it is clear that adult hippocampal neurogenesis can by dynamically regulated by number of factors (for review, see General Introduction), some neurogenic factors exert their effects on adult neurogenesis with regional specificity. For example, environmental enrichment, voluntary wheel running, and hippocampal-dependent spatial learning all selectively enhance neurogenesis in the adult hippocampus, but do not alter levels of neurogenesis in the olfactory bulb (Gould et al., 1999a; Brown et al., 2003a). In contrast, both estrogen administration and pregnancy enhance neurogenesis in both the hippocampus and olfactory bulb, perhaps because increases in hippocampal and olfactory neurogenesis are both functionally relevant for mating and/or maternal behavior (Tanapat et al., 1999; Smith et al., 2001; Shingo et al., 2003; Pawluski and Galea, 2007). Experiment 2 is a first step toward investigating whether prenatal choline availability's effects on basal neurogenesis express some regional specificity. If the effects of prenatal

choline on adult neurogenesis are specific to the hippocampus, it may point to mechanisms of prenatal choline's modulation of neurogenesis that are region-specific and help us understand whether prenatal choline is altering a specific population of cells during early development. Alternately, if prenatal choline's effects on adult neurogenesis are expressed in other neurogenic regions of the adult brain, this may suggest another mechanism is likely being affected by altered prenatal choline availability, such as the cholinergic system, which has been shown to regulate adult neurogenesis in both the adult hippocampus and olfactory bulb (Cooper-Kuhn et al., 2004; Kaneko et al., 2006).

In addition to the hippocampus, the olfactory bulb is the second undisputed site for continuing neurogenesis in the adult brain (Altman and Das, 1965; Lois and Alvarez-Buylla, 1994; Alvarez-Buylla and Garcia-Verdugo, 2002). The prevalence of olfactory neurogenesis has been confirmed across several mammalian species, including rodents (Altman and Das, 1965), non-human primates (Bernier et al., 2002), and humans (Bedard and Parent, 2004). Newborn cells, arising from neural stem cells in the subventricular zone (SVZ) lining the lateral ventricles (Altman and Das, 1965; Lois and Alvarez-Buylla, 1994; Alvarez-Buylla and Garcia-Verdugo, 2002), differentiate into one of two types of interneurons: granule cells and periglomerular interneurons. Granule cells comprise the great majority of adult-born olfactory neurons and are observed throughout the granule cell layer (GCL), while a small fraction are periglomerular interneurons found within the glomerular layer (Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002; Lledo and Saghatelyan, 2005). Both classes of interneurons function to modulate synaptic signaling of projection neurons in the olfactory bulb via lateral inhibitory and excitatory circuits

(for review, see Whitman and Greer, 2009). After birth in the SVZ, newborn neuroblasts migrate in chains along the rostral migratory stream (RMS) according to local signals within the RMS environment (for review, see Whitman and Greer, 2009). By five days, newborn cells start to reach the GCL of the olfactory bulb (Petreanu and Alvarez-Buylla, 2002), and can be detected in the glomerular layer by at least 10 days (Winner et al., 2002). Between 15 and 45 days, about half of all newborn cells undergo apoptotic cell death, but the remaining cells can survive for up to at least 19 months (Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002). Adult-born olfactory neurons become synaptically integrated (for review, see Whitman and Greer, 2009) and express immediate early genes in response to novel odors (Carlen et al., 2002; Magavi et al., 2005), suggesting that adult-born olfactory neurons actively participate within a mature olfactory network and may make a functional contribution. Indeed, others have implicated a role for adult olfactory neurogenesis in tasks of olfactory discrimination of perceptually similar odorants (Gheusi et al., 2000; Moreno et al., 2009), olfactory perception (Grabiec et al., 2009), and odor memory (Rochefort et al., 2002; Breton-Provencher et al., 2009; Veyrac et al., 2009).

As previously mentioned, basal forebrain cholinergic input regulates adult neurogenesis in both the olfactory bulb and the hippocampus. For example, lesions to cholinergic basal forebrain neurons in the adult brain decrease numbers of newborn neurons observed in both the olfactory bulb and dentate gyrus (Cooper-Kuhn et al., 2004; Mohapel et al., 2005), the survival of newborn neurons in both regions is enhanced by systemic administration of cholinergic agonists (Mohapel et al., 2005; Kaneko et al., 2006; Kotani et al., 2006; Kotani et al., 2008; Itou et al., in press), and adult-born neurons in the olfactory bulb and the dentate gyrus express various acetylcholine (Ach) receptor subtypes and make contact with cholinergic fibers (Mohapel et al., 2005; Kaneko et al., 2006). Unlike progenitor cells in the adult dentate gyrus (Itou et al., in press), however, cell proliferation in the SVZ is not modulated by cholinergic input (Cooper-Kuhn et al., 2004; Kaneko et al., 2006).

Cholinergic regulation of adult neurogenesis is of particular interest because prenatal choline availability during ED 12-17, a period during which basal forebrain cholinergic neurons are undergoing neurogenesis (Semba and Fibiger, 1988), can developmentally program basal forebrain cholinergic function and cholinergic neurotransmission in the adult brain (Blusztajn et al., 1998; Williams et al., 1998; Cermak et al., 1999; McKeon-O'Malley et al., 2003; Meck and Williams, 2003; Meck et al., 2008). Given the profound effects that altered prenatal choline availability has on the septohippocampal cholinergic system, it is possible that prenatal choline's modulation of adult hippocampal neurogenesis may also similarly affect neurogenesis in another neurogenic region of the adult brain (i.e., the olfactory bulb) that also receives cholinergic input. If this is true, it might suggest a role for the basal forebrain cholinergic system as one potential mechanism for prenatal choline's effects on adult neurogenesis.

To examine these issues, Experiment 2 investigated whether prenatal choline availability (supplementation and deficiency) during ED 12-17 altered levels of SVZ cell proliferation and/or survival of newborn neurons in the adult olfactory bulb. A 7-week survival time point was chosen to evaluate numbers of newborn granule cells in the olfactory bulb. By one month after cell birth in the adult rat SVZ, a there is a peak in migration to the GCL, a peak in apoptotic cell death, and the majority of newborn cells that have migrated to the olfactory bulb have committed to a neuronal phenotype (Winner et al., 2002). Thus, a 7-week survival time point was sufficient to examine the effects of prenatal choline availability on the survival of newborn olfactory neurons. Because it is unknown, Experiment 2 also examined whether prenatal choline availability altered olfactory bulb expression levels of BDNF and NGF, neurotrophic factors that are upregulated in the adult hippocampus by prenatal choline supplementation (Experiment 1; Sandstrom et al., 2002; Glenn et al., 2007). Little is known, however, about the involvement of neurotrophic factors in olfactory bulb neurogenesis in the adult brain. Although neural stem cells and neuroblasts in the SVZ/RMS have been shown to express neurotrophin receptors (Bath et al., 2008; Galvao et al., 2008), whether or not BDNF signaling is essential for adult neurogenesis in the olfactory bulb is still equivocal (Bath et al., 2008; Galvao et al., 2008; Ga

Methods

Animals

Prenatal supplemented (SUP), control (CON), and deficient (DEF) adult female rats were produced as described in Experiment 1. Animals were a subset of animals from Experiment 1: prenatal SUP (n = 7), CON (n = 8), and DEF (n = 8) rats that were sacrificed 24 hours after the last BrdU injection (Immediate) were used to assess cell proliferation in the SVZ; prenatal SUP (n = 10), CON (n = 7), and DEF (n = 10) rats in the Home Cage group (that were sacrificed ~7 weeks after the last BrdU injection; Survival) were used to examine cell survival/neurogenesis in the olfactory bulb (see Figure 17).





Twenty-four hours or 7 weeks after the last BrdU injection, rats were anesthetized with isoflurane, decapitated, and brains were rapidly removed and midsagitally sectioned. The olfactory bulb from one half-brain of each rat was immediately removed for growth factor protein assays and stored at -80° C. The other half-brain was immediately post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 72 hours at 4° C and then cryoprotected in a 30% sucrose solution in 0.1 M PB. These half-brains were then cut coronally on the anterior tip of the corpus collasum and coronal sections of the SVZ and sagittal sections of the olfactory bulb were collected at 60 µm on a freezing microtome. Every fifth section was collected in 0.1% sodium azide in 1 M PB. An entire series of sections for all rats was processed for BrdU peroxidase immunohistochemistry

for subsequent cell counting, and representative sections from a subgroup of rats from each prenatal diet group (n = 4 per group) were processed for BrdU and NeuN dual immunofluorescence.

Immunohistochemistry

Immunohistochemistry procedures for BrdU and dual immunofluorescence procedures for BrdU and NeuN were performed as described in Experiment 1.

Quantification of BrdU double-labeled cells using unbiased stereology

BrdU+ cells in the subventricular zone (SVZ) of rats sacrificed 24 hours after the last BrdU injection and in the granule cell layer (GCL) of the olfactory bulb from rats sacrificed 7 weeks after the last BrdU injected were counted using the optical fractionator method (West, 1993; West, 1999; Mouton, 2002). Because majority (i.e., \geq 95%) of newborn neurons in the olfactory bulb differentiate into granule cells (Winner et al., 2002; Lledo and Saghatelyan, 2005) and there was very little labeling in the glomerular layer across majority of rats in the current experiment, the glomerular layer was not examined. Every fifth section was sampled for BrdU+ cells in a total of 6 sections per rat and StereoInvestigator (Microbrightfield Inc., Williston, VT) was used to sample systematically through the designated counting regions and count numbers of immunolabeled cells. An 80 x 80 μ m counting frame and a 150 μ m \times 150 μ m sampling grid for the SVZ and a 400 μ m × 400 μ m sampling grid for the olfactory bulb were used. These parameters ensured adequate sampling of BrdU+ cells through the SVZ and olfactory bulb. Gundersen coefficient of errors were ≤ 0.09 for all optical fractionator estimates, with a range of 0.04 to 0.09. The GCL of the olfactory bulb was not examined

in its entirety due to extensive variability in its anatomy from section to section, but rather anatomic landmarks (e.g., mitral cells, external and internal plexiform layers) were used to define GCL regions, a procedure that has been used elsewhere (Cooper-Kuhn et al., 2004). For analysis of BrdU+ cells, an optical dissector height of 20 µm with a 2-µm guard zone was used to avoid over-sampling and stained cells were counted in each frame using a 40x objective lens. Optical fractionator estimates were multiplied by 2 to account for both hemispheres. Estimates of the volume of SVZ and olfactory bulb that was sampled for BrdU counting estimates were made using Cavalleri's principle (Mouton, 2002). For each section examined, the area of the region of interest was calculated by the StereoInvestigator software and was based on the boundaries of the contour tracings. Volume estimates were obtained by multiplying the section area estimates with the spacing between sampled areas. Spacing was derived by multiplying the measured, posthistology thickness of each sample (which was constant for all rats; ~50% shrinkage) by the number of sections examined.

Analysis of BrdU+ cells for NeuN co-expression using confocal microscopy

At least 50 BrdU+ cells in the GCL of the olfactory bulb of a subgroup of SUP, CON, and DEF (n = 4 per group) rats were individually examined for the co-expression of the mature neuronal marker NeuN using a Zeiss Axio Observer inverted confocal laser-scanning microscope equipped with LSM 510 software. BrdU+ cells were analyzed using z-sectioning at 0.5 µm intervals at 40x. Percentages of BrdU+ cells that coexpressed NeuN were individually calculated for each rat analyzed and then multiplied by the total number BrdU+ cells for each rat to yield an estimated number of new neurons in the GCL of the olfactory bulb.

ELISA for BDNF and NGF

Using whole olfactory bulb tissue extracts, BDNF and NGF ELISA procedures were performed as described in Experiment 1.

Data analyses

The number of BrdU+ cells in the SVZ estimated with the optical fractionator was used for statistical analyses. However, because the GCL of the olfactory bulb was not examined in its entirety, but rather anatomic landmarks were used to define GCL regions for unbiased stereological counting, there was considerable variability between rats in olfactory bulb volume estimates used for stereological counting. Thus, the estimated number of BrdU+ cells in the olfactory bulb (generated from the optical fractionator) / unit of volume (um³) was used as the primary measure for statistical analysis. Volume estimates were made using the optical fractionator and according to Cavalleri's principle (see Methods). Olfactory bulb expression levels of BDNF and NGF were expressed as percent of control levels. All of preceding measures were subjected to a one-way (Diet: SUP vs. CON vs. DEF) between-subjects ANOVA, and were followed up with Fisher's post-hoc tests when appropriate. Of particular interest were differences between CON and SUP rats, given prenatal choline supplementation's effect on hippocampal neurogenesis. Thus, a priori comparisons were used to evaluate differences between CON and SUP rats. A significance level of 0.05 was set for all statistical tests. Values are reported in the text as means \pm SEM. In accordance with Experiment 1, subjects

contained within each experimental condition were randomly selected from different litters (n of 1/litter). Thus, necessary precautions were taken to be sure that the findings were not contaminated by a lack of within-litter variability.

Results

Prenatal choline availability does not alter subventricular zone cell proliferation in the adult rat brain

To examine the addition of newly dividing cells in the SVZ that occurred over 4 days of BrdU administration, SUP, CON, and DEF rats were sacrificed 24 hours after the last BrdU injection and the SVZ of lateral ventricle wall of each rat was examined for cells immunopositive for BrdU. All three prenatal diet groups showed a high density of BrdU labeling throughout the rostral-caudal extent of the SVZ in both the dorsal and ventral regions, with no apparent differences between diet groups (Figure 18A-C). This observation was confirmed by statistical analysis of estimates of the number of BrdU+ cells in the SVZ that were generated for each rat, using unbiased stereology. SUP, CON, and DEF rats had similar numbers of BrdU+ cells in the SVZ (F < 1; Figure 18D), and there were no significant differences between diet groups in estimated volume of the SVZ (F < 1).

Prenatal choline supplementation enhances survival of newborn neurons in the adult rat olfactory bulb

To examine whether prenatal choline availability alters the long-term survival of newborn cells (that originated from the SVZ) observed within the olfactory bulb, a separate group of prenatal SUP, CON, and DEF rats were sacrificed 7 weeks after the last BrdU injection. There was clear BrdU labeling in the GCL of the olfactory bulb in all





Figure 18. BrdU Immunolabeling and Quantification in the SVZ 24 Hours After BrdU Administration. SUP (A), CON (B), and DEF (C) rats show similar densities of BrdU labeling throughout the SVZ. (D) Unbiased stereological counts of BrdU+ cells in the SVZ confirmed that SUP, CON, and DEF rats had statistically similar levels of BrdU+ cells in the SVZ. Bars in photomicrographs indicate 50 µm. Photomicrographs in top row were taken with a 10x objective and photomicrographs in the bottom row were taken with a 40x objective. LV, lateral ventricle.

prenatal diet groups, but SUP rats tended to show a higher density of BrdU labeling in the olfactory bulb than CON and DEF rats (Figure 19A-C). This was confirmed by a oneway ANOVA on the number of BrdU+ cells/ μ m³, which revealed a main effect of Diet, F(2, 27) = 4.95, p < 0.05. Post-hoc tests confirmed that SUP rats had a significantly higher number of BrdU+ cells/ μ m³ than both CON and DEF rats (ps < 0.05), while CON and DEF rats showed similar levels of BrdU+ cells/ μ m³ in the olfactory bulb (Figure 19D). Although there was considerable variability between rats in olfactory bulb volume estimates, estimates of the number of BrdU+ cells in the olfactory bulb, based on the fraction of tissue that was sampled, also revealed the same pattern of findings (Figure 19E). In contrast to SVZ cell proliferation where prenatal choline availability had no effect on the number of BrdU+ cells, prenatal choline supplementation appears to enhance long-term cell survival of these cells once they migrate to the olfactory bulb.

Newly generated BrdU+ cells in the olfactory bulb were examined in a subgroup of SUP, CON, and DEF rats (n = 4 per group, ≥ 50 cells per rat) for the co-expression of NeuN (mature neuronal marker) using dual immunofluorescence and confocal analysis (Figure 20). Confocal analysis of BrdU+ cells revealed that the majority of BrdU+ cells found in the GCL of the olfactory bulb 7 weeks after BrdU administration were also immunopositive for the mature neuronal marker NeuN (SUP: 83.70 ± 1.66%; CON: 85.42 ± 1.57%; DEF: 80.68 ± 1.54%) with no effect of Prenatal Diet (F = n.s.), indicating that majority of these newborn cells in the GCL were olfactory bulb granule cells across all treatment groups. Although there was no effect of prenatal diet on the relative proportion of neuronal differentiation in the olfactory bulb, these data, taken together







Figure 20. Phenotypic Analysis of BrdU+ Cells in the GCL of the Olfactory Bulb. Confocal images showing examples of BrdU+ cells co-labeled with the mature neuronal marker NeuN (arrows) or neither (arrow heads). Scale bars indicate 25 µm. Confocal images taken with a 40x objective. with the BrdU findings, confirm that prenatal choline supplementation enhances olfactory bulb neurogenesis.

Prior work has suggested olfactory bulb input may influence basal levels of adult hippocampal neurogenesis, as olfactory bulbectomy in adult rats decreases neurogenesis in the dentate gyrus (Jaako-Movits and Zharkovsky, 2005). Although the current experiment could not test a direct causal relation between basal levels of neurogenesis between these two neurogenic regions in the adult brain, because the current experiment utilized a subgroup of rats from Experiment 1 that had BrdU+ cell counts generated from the dentate gyrus, this afforded the opportunity to correlate olfactory bulb neurogenesis with hippocampal neurogenesis in the same group of animals. There was a strong, significant correlation between the number of BrdU+ cells/ μ m³ in the olfactory bulb and the number of BrdU+ cells in the SGZ-GCL of the dentate gyrus observed 7 weeks after BrdU administration, r(27) = 0.42, R² = 0.17, p < 0.05 (Figure 21). These findings suggest that dentate cell survival is correlated with olfactory bulb survival in the adult brain and that prenatal diet has similar effects on the capacity for adult neurogenesis across neurogeneic regions in the adult brain to some degree.

Prenatal choline availability does not alter olfactory bulb expression of BDNF or NGF in adulthood

To examine whether altered prenatal choline availability would similarly influence expression levels of neurotrophic factors in the olfactory bulb as has been previously observed in the hippocampus, protein levels (expressed as percent of control values) of BDNF and NGF were quantified in SUP, CON, and DEF rats that were



Figure 21. Correlation Between Olfactory Neurogenesis and Hippocampal Neurogenesis in the Adult Brain. A plot of number of BrdU+ cells/ μ m³ in the GCL of the olfactory bulb vs. the number of BrdU+ cells in the SGZ-GCL of the dentate gyrus observed 7 weeks after BrdU adminstration for individual SUP (red circles), CON (blue squares), and DEF (grey triangles) rats shows a significant moderate correlation, r(27) = 0.42, R² = 0.17, p < 0.05.



Figure 22. Adult Olfactory Bulb Neurotrophic Factor Expression. SUP, CON, and DEF rats had statistically similar expression levels of both BDNF and NGF in the olfactory bulb.

sacrificed ~7 weeks after BrdU administration and are presented in Figure 22. Analyses revealed no effect of Diet for either BDNF or NGF (Fs < 1; Figure 22), indicating that prenatal choline availability did not significantly alter expression of either neurotrophic factor within the olfactory bulb.

Discussion

Experiment 2 showed that prenatal choline supplementation during ED 12-17 increased adult neurogenesis in the olfactory bulb by promoting the survival of newborn granule cells, but not by altering proliferation of cells in the SVZ. In contrast, prenatal choline deficiency had no effect on either SVZ cell proliferation or on olfactory neurogenesis. Together with the results of Experiment 1, these findings indicate that prenatal choline's effects on adult neurogenesis are not regionally specific. Rather, altered prenatal choline availability exerts parallel effects on adult neurogenesis in two distinct neurogenic regions in the adult brain: in the same group of animals, prenatal choline supplementation enhanced basal levels of adult neurogenesis in the hippocampus (Experiment 1) and in the olfactory bulb (Experiment 2), while prenatal choline deficiency had little or no effect on basal neurogenesis in either region.

Whereas prenatal choline supplementation increases the proliferation and supports the survival of newborn neurons in the adult dentate gyrus (Experiment 1; Glenn et al., 2007; Glenn et al., 2008b; Wong-Goodrich et al., 2008a), Experiment 2 demonstrated that supplementation enhanced the survival of newborn neurons in the olfactory bulb, but did not affect cell proliferation in the SVZ. Taken together, these findings may point to the basal forebrain cholinergic system as one potential mechanism for prenatal choline's effects on adult neurogenesis, as SVZ cell proliferation does not appear to be regulated by cholinergic input. Similar to the current findings, systemic administration of cholinergic agonists (i.e., acetylcholinesterase inhibitors) increases the survival of new adult-born neurons in the olfactory bulb (Kaneko et al., 2006), and the proliferation and survival of new cells/neurons in the adult hippocampus (Mohapel et al., 2005; Kaneko et al., 2006; Itou et al., in press), with no effect on cell proliferation in the SVZ (Mohapel et al., 2005; Kaneko et al., 2006). In addition, neurotoxic lesioning of basal forebrain cholinergic neurons also has no effect on SVZ cell proliferation, but dramatically reduces adult neurogenesis in both the olfactory bulb and dentate gyrus (Cooper-Kuhn et al., 2004; Mohapel et al., 2005).

Prenatal choline supplementation increases the soma size of basal forebrain cholinergic neurons that project to the hippocampus (Williams et al., 1998) and enhances cholinergic transmission in the septohippocampal pathway in adulthood (i.e., increased Ach release, larger Ach pool, and slower Ach turnover in the hippocampus) (Blusztajn et al., 1998; Meck et al., 2008). Enhanced cholinergic transmission in prenatally choline supplemented rats may act directly on adult-born neurons in the dentate gyrus and olfactory bulb. Indeed, both immature neurons and newborn mature neurons in the olfactory bulb and dentate gyrus (and neural stem cells from the SGZ of the dentate gyrus) express various Ach receptor subtypes (Mohapel et al., 2005; Kaneko et al., 2006; Itou et al., in press) and thus have the ability to directly respond to cholinergic input. Although it is unknown whether stem/progenitor cells in the SVZ express Ach receptors, unlike the olfactory bulb and dentate gyrus, which make contact with cholinergic fibers, the SVZ is devoid of cholinergic innervation (Kaneko et al., 2006). Thus, a lack of effect of prenatal choline supplementation on SVZ cell proliferation is in line with previous studies demonstrating the regulation of neurogenesis by the cholinergic system, and may be a key finding to support the role for the cholinergic system as a mechanism by which prenatal choline enhances adult neurogenesis.

Future studies are needed, however, to determine whether prenatal choline availability alters basal forebrain cholinergic neurons that project to the olfactory bulb, or changes cholinergic transmission in the olfactory bulb itself. In the rat brain, the majority of the basal forebrain cholinergic neurons that project to the olfactory bulb originate from the horizontal limb of the diagonal band of Broca, with a small portion arising from the vertical limb (Rye et al., 1984; Woolf et al., 1984). Cholinergic neurons that project to the hippocampus originate from the medial septum as well as the vertical limb (Rye et al., 1984; Woolf et al., 1984). Although the forebrain cholinergic neurons that project to the hippocampus and olfactory bulb originate from these different regions, the vertical and horizontal limbs lie adjacent to each other and do not follow classical neuroanatomical boundaries, and developmental neurogenesis of basal forebrain cholinergic neurons within each limb follows relatively the same pattern of development between ED 12-17, with peaks of neurogenesis occurring on the same embryonic day (Semba and Fibiger, 1988). Therefore, it may be reasonable to predict that in addition to the septohippocampal pathway, prenatal choline supplementation during ED 12-17 would similarly enhance the cholinergic projection to the olfactory bulb in adulthood as well.

Experiment 2 also revealed that, unlike the hippocampus, prenatal choline supplementation's enhancement of olfactory bulb neurogenesis was not associated with an increase in the expression of BDNF and NGF in the adult olfactory bulb. The role of neurotrophic/growth factor expression in olfactory neurogenesis (particularly within the olfactory bulb itself) is not well understood. Although some work has suggested that BDNF within the SVZ may be important for adult SVZ/olfactory bulb neurogenesis (Benraiss et al., 2001; Bath et al., 2008), recent evidence suggests that BDNF signaling is not essential for olfactory neurogenesis (Galvao et al., 2008), and that increased BDNF delivery to the SVZ may actually decrease olfactory neurogenesis in the adult rat (Galvao et al., 2008). No study to date has examined the specific role of NGF in adult olfactory bulb neurogenesis, and few studies have attempted to address the role of neurotrophic/growth factor expression within the olfactory bulb (and not the SVZ), as was done in the current experiment. It may be that neurogenesis within the olfactory bulb itself (and its modulation by prenatal choline) depends more on alternate mechanisms shown to regulate survival of newborn neurons within the adult olfactory bulb, such as cholinergic signaling (Mohapel et al., 2005; Kaneko et al., 2006; Itou et al., in press) and CREB activation (Giachino et al., 2005), both of which are enhanced by prenatal choline supplementation in the adult brain (Blusztajn et al., 1998; Mellott et al., 2004; Meck et al., 2008).

The results of the current experiment stimulate avenues of future research investigating the functional contribution of increased numbers of newborn olfactory neurons in prenatally choline supplemented rats. For example, enhanced olfactory neurogenesis in the adult brain by prenatal choline supplementation may confer some benefit for olfactory function in prenatally choline supplemented rats, like olfactory discrimination of perceptually similar odorants (Gheusi et al., 2000; Moreno et al., 2009), olfactory perception (Grabiec et al., 2009), and odor memory (Rochefort et al., 2002; Breton-Provencher et al., 2009; Veyrac et al., 2009). Future studies are needed to examine this possibility, and whether enhanced olfactory function would also contribute to improved performance on other cognitive tasks (Meck and Williams, 2003; McCann et al., 2006).

EXPERIMENT 3: PRENATAL CHOLINE SUPPLEMENTATION ATTENUATES HIPPOCAMPAL RESPONSE TO SEIZURES IN ADULTHOOD¹

Increased availability of the nutrient choline during embryonic days (ED) 12-17 of the rat gestational period leads to improvements in memory function during young, middle, and late adulthood on hippocampally mediated tasks, (Experiment 1; Meck et al., 1988; Meck et al., 1989; Meck and Williams, 1997b; Williams et al., 1998; Meck and Williams, 2003; Meck et al., 2008; Wong-Goodrich et al., 2008a). Remarkably, prenatal choline supplementation has also been shown to protect young adult rats from seizureinduced spatial memory retention deficits normally observed after excitotoxic seizures (Yang et al., 2000; Holmes et al., 2002). While the mechanism(s) by which prenatal choline supplementation confers neuroprotection is not fully understood, choline is a vital nutrient important for several biological functions: acetylcholine synthesis, building biological membranes, cell signaling, and methyl donation (Blusztajn, 1998; Zeisel, 2004; Zeisel, 2006). One possible proximate mechanism may be enhanced adult hippocampal plasticity and neurotrophic support prior to injury. For example, prenatal choline supplementation augments several features of adult hippocampal plasticity known to influence learning and memory function, such as increased baseline levels of neurogenesis, (Glenn et al., 2007), a reduced threshold to induce LTP (Pyapali et al., 1998; Jones et al., 1999), increased dendritic spine density in area CA1 and dentate gyrus (Meck et al., 2008), and enhanced depolarization-induced MAPK and CREB activation

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(Mellott et al., 2004). Prenatal choline supplementation also enhances several neurotrophic factors in the adult hippocampus that both support plasticity (e.g., neurogenesis, LTP, etc.) and that are neuroprotective, such as BDNF, NT-3, VEGF (Glenn et al., 2007; Glenn et al., 2008b), NGF (Sandstrom et al., 2002), and IGF-1 and -2 (Napoli et al., 2008; Wong-Goodrich et al., 2008b). Enhanced hippocampal plasticity and a larger pool of neuroprotective growth factors may underlie prenatal choline supplementation's neuroprotection of the hippocampus, rendering the hippocampus better able to withstand or recover from the deleterious effects of a neural insult, such as seizures.

Status epilepticus (SE), a period of prolonged seizures, produces a host of plastic changes in the hippocampus that are thought to contribute to the development of temporal lobe epilepsy. SE results in substantial neuronal loss (e.g., Cavazos et al., 1994; Haas et al., 2001; Gorter et al., 2003), γ-aminobutyric acid (GABA) system alterations (Houser and Esclapez, 1996; Morimoto et al., 2004; Sperk et al., 2004; Brooks-Kayal et al., 2009), reactive gliosis (e.g., Jorgensen et al., 1993; Niquet et al., 1994a; Kang et al., 2006), mossy fiber innervation of the dentate gyrus (Sutula et al., 1988; Ben-Ari and Represa, 1990), changes in levels of growth factors (Khrestchatisky et al., 1995; Mudo et al., 1996; Schmidt-Kastner et al., 1996; Shetty et al., 2004), and a transient increase in cell proliferation and neurogenesis (Bengzon et al., 1997; Parent et al., 1997; Scharfman et al., 2000; Hattiangady et al., 2004). These SE-induced degenerative and regenerative changes in the hippocampus are also accompanied by deficits in hippocampal-dependent learning and memory (Stafstrom et al., 1993; Liu et al., 1994; Sarkisian et al., 1997; Hort

et al., 1999; Mikati et al., 2001). Understanding the relationship between the hippocampal response to SE and its cognitive consequences may lead to new insights into the treatment of SE and epilepsy.

Given that prenatal choline supplementation has been shown to protect against seizure-induced memory loss on a hippocampal-dependent task (Yang et al., 2000; Holmes et al., 2002), it was hypothesized that prenatal choline supplementation alters the neurophysiological response of the hippocampus to SE. To investigate this, Experiment 3 used a model of excitotoxic injury to examine whether prenatal choline supplementation modulates a variety of markers known to change shortly after SE, including hippocampal histopathology, glutamic acid decarboxylase (GAD) expression, dentate cell proliferation, neurogenesis, astrogliosis, and growth factor content.

Methods

Animals

Twenty timed-pregnant Sprague-Dawley rats (CD strain, Charles River, Kingston, NY) were obtained on day 9 of gestation (ED9). All dams were individually housed in clear polycarbonate cages (27.9×27.9×17.8 cm) that were individually ventilated, and the colony was maintained at 21°C on a 12-h light/dark cycle with lights on at 7 a.m. Dams were fed a control diet *ad libitum* (AIN76-A from Dyets, American Institute of Nutrition, ICN, Nutritional Biochemical, Cleveland, Ohio; 1.1 g/kg choline chloride substituted for choline bitartrate). Prenatal diet treatments encompassed the same timeline as Experiments 1 and 2, and were the same as those used in studies showing memory enhancing and memory protecting effects of prenatal choline supplementation (Meck et

al., 1988; Meck et al., 1989; Yang et al., 2000; Holmes et al., 2002; Meck and Williams, 2003). On the morning of ED11 to the morning of ED18 (ED 11-17), pregnant dams were either given *ad libitum* access to a control diet (n = 14) or a choline supplemented diet (n = 6). Control dams were given the AIN76-A diet containing 7.9 mmol/kg choline chloride and water sweetened with 50 mM saccharine, resulting in an average daily choline intake of 0.59 mmol/kg/day. Choline supplemented dams received the AIN76-A diet containing 7.9 mmol/kg choline chloride and water containing 25 mM choline chloride and sweetened with 50 mM saccharine, resulting in an average daily choline intake of 3.46 mmol/kg/day (approximately 6.8 times more choline than the control diet). On ED18, all dams were returned to normal drinking water. There were no significant differences in the amount of water intake, food consumed, or body weights on ED11-18 between control and choline supplemented dams (ps > 0.05; data not shown). After birth, offspring from the control and choline supplemented dams were toe clipped for identification and then were selected randomly and cross-fostered to dams that consumed the control diet throughout pregnancy to yield 10 pups per litter (5 males and 5 females, half from different control dams and half from supplemented dams). There were no significant differences between control and prenatally choline supplemented litter size or pup birth weights (ps > 0.05). On postnatal day (P) 25, pups were weaned and pairhoused with a rat of the same sex and prenatal diet condition. All offspring were given ad *libitum* access to the control diet through the duration of the study. Male offspring were used as subjects. All animal procedures were in compliance with the Institutional Animal Care and Use Committee of Duke University.

Induction of status epilepticus and bromodeoxyuridine injections

Figure 23 presents the timeline of experimental procedures. Status epilepticus procedures were adapted from previous reports and were based on a model of chronic temporal lobe epilepsy that yields a low mortality rate and mimics several aspects of human temporal lobe epilepsy (Hellier et al., 1998; Hellier and Dudek, 1999; Hattiangady et al., 2004; Hellier and Dudek, 2005). Kainic acid (KA) was obtained from Ocean Produce International (Shelburne, Nova Scotia, Canada) and was dissolved in 0.9% sterile saline (Sigma, St. Louis, MO). On P60, a group of adult male control offspring (CON, n = 11) and prenatally choline supplemented offspring (SUP, n = 11) were injected with KA (2.5 mg/kg, i.p.) every hour and observations of seizure behavior were recorded according to Racine's scale (Racine, 1972). A separate group of CON (n = 4) and SUP (n = 4) male offspring was similarly treated with hourly injections of 1 ml/kg saline. All KA-treated animals initially showed wet dog shakes and head-nodding, followed by Class III (unilateral forelimb clonus), Class IV (bilateral forelimb clonus with rearing), and Class V (bilateral forelimb clonus with rearing and falling over) motor seizures. KA treatment continued until rats displayed at least one Class IV/V seizure per hour for 3 consecutive hours, although most rats exhibited many more seizures during these 1-hour periods and all rats eventually entered a state of continuous Class III-V seizures for over 3 hours. If a rat displayed ≥ 10 class IV/V seizures in an hour, administration of the next injection was delayed to the next hour. If a rat displayed bouncing seizures after reaching status epilepticus, it was given a low dose of diazepam (2.5 mg/kg) to reduce the risk of mortality. We have observed and others have

demonstrated (Scharfman et al., 2000; Pitkanen et al., 2005) that administering a low dose of diazepam following status epilepticus does not compromise the development of spontaneous epileptic motor seizures. The total KA dosage administered was titrated for each rat according to each rat's motor seizure activity. Because it has been found that seizures, as opposed to neuronal cell death *per se*, lead to increases in hippocampal cell proliferation and neurogenesis (Parent et al., 1997; Tooyama et al., 2002; Smith et al., 2005), these procedures were designed such that all KA-treated rats experienced similar duration and severity of seizure activity (see Results).

After KA treatment, rats were injected subcutaneously with 5 ml of lactated Ringer's solution (Abbott Laboratories, Chicago, IL) to prevent dehydration and were monitored closely until all seizure activity subsided. Saline-treated rats were also given 5 ml of lactated Ringer's solution to equate their post-treatment experience to that of KAtreated rats. For the next 3 to 5 days, all rats continued to receive daily subcutaneous injections of 1-5 ml of lactated Ringer's solution and were provided with moistened chow and slices of fresh fruit to aid recovery. Mortality rates did not differ significantly between CON rats (2/11) and SUP rats (3/11), χ^2 (1, N = 22) = 0.26, p = 0.61.

Five days after being treated with KA or saline, all rats were administered daily injections of 5-bromo-2-deoxyuridine (BrdU; 100mg/kg/day, i.p.; Sigma, St. Louis, MO) for 10 consecutive days to label dividing cells. This injection regimen was based on past research designed to capture the impact of a variety of manipulations on cell proliferation and survival in the hippocampus (Kempermann et al., 1997b; Lee et al., 2002b; Rao et

al., 2005; Glenn et al., 2007), and to parallel the time point that prenatal choline protects against seizure-induced memory deficits (Yang et al., 2000; Holmes et al., 2002).



Figure 23. Experiment 3 Timeline of Experimental Procedures.

Tissue harvesting and cresyl violet staining

Twenty-four hours after the last BrdU injection, rats were given an overdose of a ketamine/xylazine cocktail, decapitated, and brains were rapidly removed and midsagitally sectioned. The selection of this particular time point (16 days post-KA/saline treatment) was based on 1) the findings reported in Hattiangady et al. (2004) that showed robust increases in hippocampal neurogenesis 16 days following an analogous KA treatment, and 2) prenatal choline supplementation's protection against memory deficits was observed 1-2 weeks after excitotoxin-induced status epilepticus (Yang et al., 2000; Holmes et al., 2002), which approximates the current time window chosen in this study. The entire hippocampus from one half-brain was immediately dissected out and divided into pieces of approximately equal length along the rostral-caudal extent of the hippocampus. Pieces for reverse transcriptase PCR assays (see Reverse transcriptase PCR section) were immediately homogenized in cold guanidine isothiocyanate solution, frozen on dry ice, and stored at -80° C for. The remaining pieces were immediately stored at -80° C until assayed for protein. The other half-brain from each rat was immediately postfixed in 4% paraformaldehyde for 72 hours at 4° C and then cryoprotected in a 30%

sucrose solution in 1M phosphate buffer (PB). These half-brains were then sectioned coronally at 60 µm on a microtome through the rostral-caudal extent of the hippocampus and every fifth section was collected in 0.1% sodium azide in 1M PB to yield five series of 15-20 sections each. The first and second series were processed for BrdU and doublecortin (DCX) immunohistochemistry, respectively, for subsequent cell counting. Representative samples of four to five 60 µm sections that captured the rostral-caudal extent of the hippocampus were randomly selected from the third series and processed for cresyl violet staining.

Sections stained for cresyl violet were analyzed and scored for lesion severity by 2 observers blind to the treatment conditions. The following scale, adapted from Yang et al. (2000) and Holmes et al. (2002), was used to characterize each rat's hippocampal cytopathology: a score of 0 indicated no cell loss, a score of 1 indicated minimal cell loss in CA1, CA3, and/or hilar regions, a score of 2 indicated moderate cell loss with relatively normal general cellular architecture, and a score of 3 indicated severe cell loss with considerable disruption of the general cellular architecture.

BrdU immunohistochemistry

One series of free-floating sections were processed for BrdU immunohistochemistry. Procedures for BrdU-labeling were as described in Experiment 1.

Doublecortin immunohistochemistry

An adjacent series of tissue sets was processed for DCX immunohistochemistry. These procedures were based on the methods of Rao & Shetty (2004). Free-floating sections were rinsed in TBS, treated for 30 min in 50% methanol, treated for 30 min in 0.6% hydrogen peroxide, incubated in 0.1% TTX and 3% normal horse serum in TBS for 30 min at room temperature, and then incubated with the primary antibody (affinity purified polyclonal goat antibody raised against a peptide mapping at the carboxy terminus of human DCX, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) for 4 hours at room temperature. Following this, the tissue was rinsed with TBS and incubated with the secondary antibody (biotinylated horse anti-goat, 1:200; Vector Laboratories) for 2 hours at room temperature. The tissue was then rinsed in TBS, incubated in an avidinbiotinylated peroxidase complex (ABC, Vector Laboratories) for 1 hour at room temperature, rinsed again in TBS, and developed with vector grey substrate (Vector Laboratories). Stained sections were mounted on gelatin-coated slides, dehydrated, and coverslipped.

Quantification of BrdU- and DCX-labeled cells using unbiased stereology

BrdU- and DCX-labeled cells in each dentate gyrus were counted using the optical fractionator method (West, 1993; West, 1999; Mouton, 2002). Every fifth section was sampled throughout the rostral-caudal extent of the dentate gyrus in two sampling regions: one region included the subgranular zone (SGZ), which was designated as an approximately 2-cell thick zone between the inner rim of the granular cell layer (GCL) and the hilus, and the GCL that encompassed the suprapyramidal and infrapyramidal granule cell blades, and the other region included the hilus, which did not include the CA3c region. StereoInvestigator (Microbrightfield Inc., Williston, VT) was used to sample systematically through the designated region and count numbers of labeled cells. For counting BrdU-labeled cells, a 40 x 40 µm counting frame was used and 14-41 sites

per section were analyzed in 8 sections, yielding 146-232 frames per region for each rat. These parameters ensured adequate sampling of BrdU-labeled cells through the SGZ-GCL and the hilus. The same parameters were used for counting DCX-labeled cells with the exception of a larger counting frame (80 x 80 µm) to compensate for the more sporadic distribution of DCX-labeled cells, yielding 13-33 sites per section and 117-227 frames per region analyzed for each rat. For analysis, an optical dissector height of 20 µm with a 2-µm guard zone was set and stained cells were counted in each frame using a 40x objective lens. Finally, estimates of the volume of the regions of dentate gyrus that was sampled for BrdU and DCX estimates were made using Cavalleri's principle (Mouton, 2002). For each section examined, the area of the SGZ-GCL and hilus was calculated by the StereoInvestigator software and was based on the boundaries of the contour tracings. Volume estimates were obtained by multiplying the section area estimates with the spacing between sampled areas. Spacing was derived by multiplying the measured, post-histology thickness of each sample by the number of sections examined.

ELISA for neurotrophic and growth factors

For ELISA assays, whole tissue extracts from all dissected samples were prepared as described in Experiment 1.

The ChemiKine[™] BDNF sandwich ELISA kit (Chemicon Int., Inc.) was used to assay the BDNF levels in hippocampal lysates, according to procedures described in Experiment 1.

The Quantikine® sandwich ELISA kits (R&D Systems) were used to assay IGF-1 and FGF-2 levels in the samples. ELISAs were performed according to manufacturer's
instructions. Briefly, microplates were pre-coated with the first primary antibody (monoclonal in IGF-1 and FGF-2 kits). Assay diluents were added to each well of the microplate (50 µL/well for IGF-1 assay and 100 µL/well for FGF-2 assay). Standard control samples for each neurotrophic factor were diluted serially (1:2) from either 6–0 ng/mL (IGF-1) or 32–0 pg/mL (FGF-2) or with their respective calibrator diluents and plated to two columns of wells (50 µL/well in IGF-1 assay and 100 µL/well in FGF-2 assay) designated for standard curve in every plate. The frozen ELISA samples (described above) were thawed on ice, and every sample plated in duplicate for measurement of each of the factors. Following a 2 hour incubation at room temperature, wells were rinsed in wash buffer and treated with an enzyme-linked second primary antibody solution for 2 hours. The second primary antibody was a polyclonal IGF-1 antibody conjugated to HRP in IGF-1 detection kit or a monoclonal FGF-2 antibody conjugated to alkaline phosphatase in FGF-2 detection kit. The wells were rinsed in wash buffer and a substrate solution was added to the wells and incubated in the dark for 30 min for IGF-1 or 45 min for FGF-2. The color reaction was stopped with 1M hydrochloric acid in the IGF-1 assay or with 2N sulfuric acid in the FGF-2 assay.

The Emax® immunoassay systems (Promega) were used to measure NGF and NT-3 in the samples. Flat-bottom 96 well plates (NUNC) were first coated with solution containing a polyclonal antibody against either NGF or NT-3 (the first primary antibody solution) prepared in carbonate coating buffer (100 μ L/well, 1:1000 dilution) and incubated for 16 hour at 4°C. Following a wash in TBST (Tris-buffered saline solution containing Tween 20), the coated wells were incubated with block and sample buffer

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(1X) for 1 hour at room temperature and washed again with TBST. Standard control samples for NGF and NT-3 were diluted serially (1:2) from 250–0 pg/mL or 300–0 pg/mL, respectively, and plated in duplicate (100 μL/well). The frozen ELISA samples (described above) were thawed on ice, and every sample plated in duplicate for measurement of NGF or NT-3. Following a 6 hour incubation at room temperature, wells were washed in TBST. Diluted monoclonal antibody against either NGF or NT-3 (the second primary antibody solution; 1:4000) was added to each well and incubated overnight at 4°C. The wells were washed in TBST, incubated with appropriate secondary antibody conjugated to peroxidase for 2.5 hours, washed again in TBST, and treated with tetramethyl benzidine (TMB) substrate for 10 min. The chromogen reaction was stopped by adding 100 μL of 1N hydrochloric acid.

The optical density of each well was measured using the Victor³ microplate reader (PerkinElmer Life Sciences). The intensity of color was measured at a wavelength of 450 nm for all ELISAs. In order to correct for optical imperfections in the plate, readings at 540 nm were subtracted from readings at 450 nm. The standard curve was used to assess the validity of the protocol and to determine the relative concentrations of the growth factors. Values in all samples were normalized per gram of tissue assayed, and the average value for each sample was calculated separately before determining the group means.

Western blot analysis for GAD65, GAD67, and GFAP

For Western blot analysis, the lysates were prepared as described in the ELISA methods. However, a portion of the lysate was taken before dilution in Dulbecco's PBS.

The extracts were normalized for total protein and subjected to SDS-PAGE. After transfer of protein to an Immoblin P membrane (Millipore), the membrane was blocked with 5% nonfat dry milk in 1X Tris-buffered saline (TBS) containing 0.1% Tween 20 for 2 hours and then probed overnight with either anti-glial fibrillary acidic protein (GFAP) monoclonal antibody GA5 (1:1000) (Cell Signaling Technology), anti-GAD65/67 polyclonal antibody (1:1000) (Chemicon), or anti-β-actin monoclonal A5441 (1:5000; Sigma). The antibody/antigen complexes on the membranes were detected using a peroxidase-conjugated anti-mouse IgG for GFAP and β-actin (1:2000) or anti-rabbit IgG (1:5000) for GAD65/67 and visualized using the enhanced chemiluminescence method (Western Lightning, Perkin Elmer) and a Kodak Image Station 440. Digitized images of immunoblots were quantified using Kodak ID software. Protein levels were normalized with β-actin values.

Reverse transcriptase PCR

Total RNA was extracted from tissues by phenol and chloroform method (Chomczynski and Sacchi, 1987; Sambrook, 1989) and precipitated. RNA was resuspended and its quantity was determined using Quant-iTTM RiboGreen^{*} RNA assay kit (Molecular Probes) and the Victor³ multi-label plate reader (PerkinElmer Life Sciences). Hippocampal RNA was used for reverse transcriptase (RT)-PCR using Superscript One-Step RT-PCR with Platinum *Taq* (Invitrogen). First strand cDNA synthesis was performed with 25 ng of total RNA, oligo dT primers and reverse transcriptase at 48°C (45 min). Primers used for PCR include β -actin (Forward: CAC AGC TGA GAG GGA AAT C, Reverse: TCA GCA ATG CCT GGG TAC), DCX (Forward: CGA TCA AAC TGG AAA CCG G, Reverse: TTT GCG TCT TGG TCG TTA CC), GAD65 (Forward: TCT TTT CTC CTG GTG GTG CC, Reverse: CCC CAA GCA GCA TCC ACA T), GAD67 (Forward: TACGGGGTTCGCACAGGTC, Reverse: CCCCAAGCAGCATCCACAT), and GFAP (Forward: ACA TCG AGA TCG CCA CCT AC, Reverse: ACA TCA CAT CCT TGT GCT CC). PCR was performed using Platinum *Taq* DNA polymerase with a denaturing step for 2 min at 94°C, followed by 28-40 cycles (36 cycles for β-actin; 32, DCX; 31, GAD65; 38, GAD67; and 28, GFAP) of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, and terminated by an elongation step at 72°C for 7 min. PCR products were separated on a 10% TBE polyacrylamide gel and stained with ethidium bromide. PCR products were then visualized with the Kodak Image Station 440 (Rochester, NY) and product intensities were quantified using Kodak software. PCR product levels were normalized with β-actin values.

Data analyses

Numbers of BrdU-labeled and DCX-labeled cells estimated with the optical fractionator, the volumes of dentate gyrus that were estimated using Cavalleri's principle, and protein and mRNA levels (expressed as percent of control levels) were all subjected to a 2 (Diet: CON vs. SUP) \times 2 (Seizure: saline vs. KA) between-subjects ANOVA. Of particular interest were Diet \times Seizure interactions. Where appropriate, *a priori* pairwise comparisons were used to evaluate differences between group means. A significance level of 0.05 was set for all statistical tests. Values are reported in the text as means \pm SEM. Note that subjects contained within each experimental condition were randomly

selected from different litters (n of 1/litter). Thus, necessary precautions were taken to be sure that findings were not contaminated by a lack of within litter variability.

Results

Seizure activity and hippocampal histopathology after KA-induced SE

All rats treated with KA reached SE and exhibited continuous Class III-IV seizures for over 3 hours. Careful observation of behavioral activity of rats receiving KA revealed that the pattern of seizure activity (progression, number, and severity of seizures) was similar for both CON and SUP rats. There were no significant differences between CON and SUP rats in the number of Class III/IV/V or total number of motor seizures within the first 1-hour period of observable seizure activity (CON: Class III = 0.25 ± 0.25 , Class IV = 7.00 ± 1.41, Class V = 9.25 ± 3.61, total = 16.50 ± 4.91; SUP: Class III = 4.00 ± 1.47 , Class IV = 10.00 ± 2.35 , Class V = 8.50 ± 3.52 , total = $22.50 \pm$ 6.81; all ps > 0.05). After exhibiting the first motor seizure, all rats entered a continuous class III-V seizure state in the second 1-hour period. To ensure that differences in seizure severity or duration did not occur beyond the 3-hr time window, both CON and SUP rats continued to be monitored and no differences were apparent. All rats gradually dropped out of SE within 2-3 hours of the last injection: continuous seizures subsided, followed by the emergence of 1-3 discrete Class III/IV/V seizures within a 1-hour period, and the absence of seizure activity by 5 hours after the last injection. There were also no significant differences between CON and SUP rats in the total amount of KA needed to induce SE (range = 3.75-16.25 mg/kg) or in the latency to the first motor seizure (range = 62-331 min). This variability in dose and latency to the first behavioral seizure in our rats

is consistent with previous reports showing that the Sprague Dawley rats show a more variable convulsant response to KA than other rat strains (Golden et al., 1991; Golden et al., 1995). Thus, while the dose of KA needed to induce SE varied across rats, the SE produced was quite comparable between CON and SUP rats.

Histopathology scores adapted from a previous report showing protection against histopathology following KA treatment as a result of prenatal choline supplementation (Holmes et al., 2002) were used to confirm that the current KA treatment lead to similar patterns of histopathology between CON and SUP rats. Comparable to Holmes et al. (2002), the current experiment also found evidence of considerable histopathology from CON rats that experienced KA-induced SE, and this damage also appeared to be attenuated in SUP rats. Figure 24 shows photomicrographs of representative brain sections stained with cresyl violet from CON and SUP rats 16 days following KA treatment. While the GCL of the dentate gyrus remained intact, areas CA1, CA3, and the hilus of KA-treated rats displayed cell loss, disrupted cytoarchitecture, and gliosis. The histopathology of the hippocampus was more severe in KA-treated CON rats when compared to KA-treated SUP rats (Figure 24B, 24C). KA-treated SUP rats also had significantly lower histopathological scores than KA-treated CON rats (CON: $2.54 \pm$ 0.40, SUP: 1.44 ± 0.37 , t (6) = 2.01, p < 0.05, one-tailed). To further examine the nature of the neuropathological response to KA-induced seizures in CON and SUP rats, we quantified a number of neurobiological markers in the hippocampus known to change as a result of SE and possibly contribute to the spatial learning and memory deficits observed shortly after seizures.



Figure 24. Hippocampal Histopathology of CON and SUP Rats 16 Days After KA-Induced SE. Histopathology of the hippocampus in CON (*B*) and SUP (*C*) rats. Panel A depicts representative sections of CA1 (*A1*), CA3 (*A2*), and dentate gyrus (*A3*) regions from an intact hippocampus from a saline-treated CON rat. Saline-treated SUP rats also did not show any lesions (histology data not shown). Note more severe degeneration (cell loss, disruption of cytoarchitecture, and gliosis) in KA-treated CON rats (*B1, B2, B3*). Areas of damage are indicated by arrows. Dentate hilar cell loss was observed in both CON (*B3*) and SUP rats (*C3*). Photomicrographs in each set were taken with a 10x objective. Bars indicate 50 µm. GCL, granule cell layer. SGZ, subgranular zone. H, hilus.

Prenatal choline supplementation protects against SE-induced decrease in hippocampal GAD65, but not GAD67, mRNA and protein expression

Excitotoxin-induced SE is known to cause a decrease in inhibition (Sloviter, 1987; Cornish and Wheal, 1989; Milgram et al., 1991; Kobayashi and Buckmaster, 2003) and degeneration of GABAergic neuronal function in the hippocampus (Obenaus et al., 1993; Houser and Esclapez, 1996; Morin et al., 1998; Esclapez and Houser, 1999; Shetty and Turner, 2001), which has been considered to be a significant contributor to hyperexcitability in the epileptic hippocampus. This seizure-induced disruption in GABAergic function can be measured via quantification of GAD65 and 67, enzymes important for local GABA synthesis at synaptic (GAD65) and cytoplasmic (GAD67) sites (Erlander and Tobin, 1991; Esclapez et al., 1994), and may play a role in the packaging and release of GABA (Namchuk et al., 1997; Tian et al., 1999). Thus, as a marker of GABAergic function, levels of hippocampal GAD65 and 67 mRNA and protein (via Western blot) were examined in saline- and KA-treated CON and SUP rats 16 days post-SE and these data, expressed as percent of control levels, are shown in Figure 25. Analyses of mRNA levels revealed a significant Diet × Seizure interaction for GAD65 mRNA, F(1, 12) = 17.19, p < 0.001. Baseline GAD65 mRNA levels were 33% lower in SUP rats when compared to CON rats (p < 0.05; Figure 25B). Sixteen days following SE, GAD65 mRNA levels were reduced by more than half in CON rats (p <0.001), which is consistent with previous reports that show decreases in hippocampal GAD65 mRNA 3-14 days following seizures (Houser and Esclapez, 1996; Kobayashi and Buckmaster, 2003). In contrast, there was no significant difference in GAD65 mRNA levels between saline-treated and KA-treated SUP rats (Figure 25B). Although a Diet \times

Seizure interaction did not reach statistical significance for GAD65 protein levels, F(1, 10) = 2.57, p = 0.14, planned comparisons revealed that KA-treated CON rats showed a significant decrease by more than half in GAD65 protein (p < 0.05) whereas GAD65 protein levels did not differ significantly between KA-treated and saline-treated SUP rats (Figure 25A). Taken together, these data suggest a preservation of hippocampal GABAergic GAD65-containing neurons, or a more rapid recovery of GAD65 in SUP rats. In contrast, neither prenatal diet nor KA treatment had an effect on levels of GAD67 mRNA 16 days following SE (all Fs < 1), as levels of GAD67 mRNA did not significantly differ across the four conditions (Figure 25B). Analyses of GAD67 protein levels in both CON rats and SUP rats, F(1, 10) = 4.78, p = 0.05 (Figure 2A). SUP rats also exhibited lower levels of GAD67 protein overall, F(1, 10) = 8.43, p < 0.05. These findings suggest that prenatal choline supplementation's neuroprotective effect on GAD expression 16 days following seizures may be specific to the GAD65 isoform.

Prenatal choline supplementation attenuates SE-induced upregulation of hippocampal cell proliferation in the dentate gyrus

To detect changes in dentate cell proliferation as a result of KA-induced SE, we visualized cells immunopositive for the cell division marker, BrdU, administered 5-15 days post-SE to capture the time window during which savings in spatial learning and memory following excitotoxin-induced SE have been observed with prenatal choline supplementation (Yang et al., 2000; Holmes et al., 2002). The amount of cell proliferation after SE that was captured with the current 10-day BrdU regimen yielded a



Figure 25. Hippocampal GAD Expression 16 Days After Saline or KA Treatment in CON and SUP Rats. Comparison between CON (white bars) and SUP rats (grey bars) in GAD65 and GAD67 protein levels (A) and mRNA levels (B) (mean ± SEM percent of control levels) in the intact hippocampus (open bars) and 16 days following KA-induced SE (hatched bars). Protein levels were quantified using Western blot analysis (A). KA-treated CON rats showed a significant decrease in GAD65 protein and mRNA levels by more than half (p < 0.001), whereas GAD65 protein and mRNA levels did not significantly change in KA-treated SUP rats. GAD67 protein levels were reduced in both KA-treated CON and SUP rats, but GAD67 mRNA levels did not change in either group as a result of KA-induced SE. * statistically different from within-diet saline-treated group; # statistically different from KA-treated CON; † statistically different from saline-treated CON.

very high density of immunostaining. Stereological procedures also require relatively thick sections for unbiased cell counting, which further contributed to the high density of BrdU labeling where many BrdU+ cells following SE were tightly clustered together and layered on top of each other, making the quantification of double labeled cells unreliable. Therefore, we quantified markers of neurogenesis and gliogenesis in the hippocampus (see additional results below). Figure 26 shows photomicrographs of BrdU labeling in the dentate gyrus of representative sections from CON and SUP saline- and KA-treated rats. BrdU+ cells were expressed throughout the rostral-caudal extent of the dentate gyrus and in both the suprapyramidal and infrapyramidal blades. BrdU+ cells were evident in the SGZ, indicating that many of the cells were newly generated. BrdU+ cells were also visible in the GCL, indicating that some had survived for several days and were migrating from the subgranular zone. Moreover, a vast majority of BrdU+ cells displayed morphological characteristics of normal non-pyknotic cells (e.g., round or oval nuclei that did not appear highly condensed). In KA-treated rats, a considerable number of BrdU+ cells were also present in the hilus. BrdU labeling clearly showed that while KA treatment dramatically upregulated SGZ-GCL and hilar cell proliferation in CON rats, which is consistent with previous reports (Bengzon et al., 1997; Parent et al., 1997; Jessberger et al., 2005), the SE-induced increase in cell proliferation was significantly attenuated in SUP rats (Figure 26B, 26D). This observation was confirmed by quantification of BrdU+ cells. Using unbiased stereology, estimates of the number of BrdU+ cells were generated for both the SGZ-GCL and hilus for each rat and these data are shown in Figure 26. As expected, SE significantly increased the number of SGZ-GCL

and hilar BrdU+ cells in both CON and SUP rats (SGZ-GCL: F(1, 12) = 24.73, p < 0.001; hilus: F(1, 12) = 32.47, p < 0.001). There were no main effects of Diet on SGZ-GCL nor hilar cell proliferation but a significant Diet × Seizure interaction was found for SGZ-GCL cell proliferation, F(1, 12) = 7.91, p < 0.05, which approached statistical significance for hilar cell proliferation, F(1, 12) = 3.67, p = 0.07). Remarkably, while CON rats exhibited a 780% increase in the total number of BrdU+ cells (SGZ-GCL and hilus combined) following SE, SUP rats showed only a 207% increase. Planned comparisons revealed that KA-treated CON rats had significantly more BrdU+ cells in the SGZ-GCL than KA-treated SUP rats (p < 0.01; Figure 27), confirming that SE-induced cell proliferation in the dentate gyrus was markedly attenuated in SUP rats.

To determine whether a larger overall size of structure could account for the higher numbers of new cells detected in KA-treated CON versus KA-treated SUP rats, the volume of the SGZ-GCL and hilus was estimated for each rat using Cavalleri's principle (Mouton, 2002). However, analyses did not detect any statistically significant differences between any treatment groups (Fs < 1).

The number of immature neurons in the dentate gyrus shortly following seizures is not modulated by prenatal choline supplementation

To determine whether prenatal choline supplementation decreased the proliferation of new neurons after SE, adjacent tissue sections were used to visualize cells immunopositive for the microtubule-associated phosphoprotein, doublecortin (DCX), that is transiently expressed in newly born neurons that are still in the process of migrating and differentiating (Brown et al., 2003a; Rao and Shetty, 2004). Others have confirmed



Figure 26. BrdU Immunolabeling 16 Days After Saline or KA Treatment in CON and SUP Rats. BrdU-immunopositive cells (i.e., newly generated cells) in the SGZ-GCL and hilus 16 days following saline treatment (A, CON; B, SUP) or KA-induced SE (C, CON; D, SUP). Note that the number of BrdU-labeled cells significantly increased 16 days after SE for both diet groups, but that this seizure-induced proliferative response was markedly attenuated in SUP rats (D) in comparison to CON rats (C). Photomicrographs in the left column were taken with a 10x objective and images in the right column were taken with a 40x objective. Bars indicate 50 µm. GCL, granule cell layer. SGZ, subgranular zone. H, hilus.



Figure 27. Quantification of BrdU+ Cells in the Dentate Gyrus 16 Days After Saline or KA Treatment in CON and SUP Rats. Mean (\pm SEM) numbers of BrdU+ cells detected in the SGZ-GCL and hilus of CON (white bars) and SUP (grey bars) rats 16 days following saline treatment (open bars) or KA-induced SE (hatched bars). SE significantly increased the number of BrdU+ cells in both CON and SUP rats (*ps* < 0.05), but this increase was significantly attenuated in SUP rats (*ps* < 0.05). * statistically different from within-diet saline-treated group; # statistically different from KA-treated CON.

that DCX expression is a reliable indicator of neurogenesis in the adult brain (Couillard-Despres et al., 2005). As a secondary measure, DCX mRNA levels were also quantified via RT-PCR. Figure 28 shows photomicrographs of DCX labeling in the hippocampus of representative sections from CON and SUP saline- and KA-treated rats. DCX+ cells were visible with processes in various stages of development and were evident along the SGZ and GCL. In KA-treated rats, DCX+ cells were also detected in the hilus, which is consistent with previous studies that have shown that a portion of these newly generated neurons after SE aberrantly migrate to the hilus region (Parent et al., 1997; Hattiangady et al., 2004; Jessberger et al., 2005). In comparison to saline-treated rats many of the DCX+ neurons in KA-treated rats appeared displaced and exhibited abnormal morphological features, such as horizontally oriented cell bodies and processes (Figure 28C, 28D).

Unbiased stereological estimates of the number of DCX+ neurons in the SGZ-GCL (all rats) and in the hilus (KA-treated rats) were generated and are shown in Figure 29. As expected, SE induced a dramatic increase in the number of DCX+ neurons in both CON and SUP rats (SGZ-GCL: F(1, 12) = 11.93, p < 0.01; hilus: F(1, 12) = 34.50, p < 0.001). However, prenatal choline supplementation did not significantly diminish the proliferation of new neurons in response to SE; there was no significant main effect of Diet for both SGZ-GCL and hilar neurogenesis or Diet × Seizure interactions (Fs < 1). There was also no significant difference in the number of DCX+ neurons between saline-treated CON and saline-treated SUP rats. SE also produced a significant increase in DCX mRNA in both CON and SUP rats (F(1, 12) = 6.49, p < 0.05), but prenatal choline availability did not alter baseline or SE-induced mRNA levels of DCX (Fs < 1; Figure



Figure 28. DCX Immunolabeling 16 Days After Saline or KA Treatment in CON and SUP Rats. DCX-immunopositive neurons (i.e., newly generated neurons) in the SGZ-GCL and hilus 16 days following saline treatment (A, CON; B, SUP) or KAinduced SE (C, CON; D, SUP). The number of DCX-labeled cells significantly increased 16 days after SE for both CON (A, C) and SUP (B, D) rats. Note that in KAtreated rats of both diet groups (C, D), DCX-positive neurons were aberrantly located in the hilus and exhibited abnormal morphological features, such as horizontally oriented cell bodies and processes (arrows). Photomicrographs in the left column were taken with a 10x objective and images in the right column were taken with a 40x objective. Bars indicate 50 µm. GCL, granule cell layer. SGZ, subgranular zone. H, hilus.



Figure 29. Quantification of DCX+ Cells in the Dentate Gyrus and Hippocampal DCX mRNA Levels 16 Days After Saline or KA Treatment in CON and SUP Rats. Mean (\pm SEM) numbers of DCX+ cells (A) and mean (\pm SEM) mRNA expression levels (B) detected in the SGZ-GCL and hilus of CON (white bars) and SUP (grey bars) rats 16 days following saline treatment (open bars) or KA-induced SE (hatched bars). KA-induced SE significantly increased the number of DCX+ neurons and levels of DCX mRNA in both CON and SUP rats (ps < 0.05). Prenatal choline supplementation did not alter the number of DCX+ neurons or levels of DCX mRNA in response to KA-induced SE. * statistically different from within-diet saline-treated group.

29). These data revealed that unlike the pattern of BrdU immunolabeling, seizure-induced upregulation of DCX+ immature neurons in the dentate gyrus was not modified by prenatal choline availability.

Prenatal choline supplementation attenuates SE-induced upregulation of hippocampal GFAP protein and mRNA expression

Prior work has shown that SE triggers astroglial activity as revealed by dramatic elevations of GFAP protein and mRNA expression (Jorgensen et al., 1993; Niquet et al., 1994a). GFAP is also expressed in progenitor cells that reside in the SGZ of the dentate gyrus, give rise to new neurons, and are stimulated by seizures (Seri et al., 2001; Segi-Nishida et al., 2008), which also makes GFAP a marker of interest given that SE produces robust increases in dentate cell proliferation. Levels of hippocampal GFAP protein (via Western blot) and mRNA were quantified in saline- and KA-treated CON and SUP rats 16 days post-treatment and these data, expressed as percent of control levels, are shown in Figure 30. As predicted, CON and SUP rats exhibited a significant increase in GFAP protein and mRNA levels as a result of KA treatment (protein: F (1, 12) = 16.60, p < 0.01; mRNA: F(1, 12) = 35.37, p < 0.05). More importantly, there was a significant Diet × Seizure interaction for GFAP mRNA levels, F(1, 12) = 5.88, p < 0.05, although a Diet × Seizure interaction for GFAP protein levels did not reach statistical significance, F(1, 12) = 2.83, p = 0.12. Saline-treated SUP rats had significantly lower levels of hippocampal GFAP protein and mRNA than CON rats (ps < 0.05; Figure 30). However, SE-induced increases in GFAP mRNA were significantly reduced in KAtreated SUP rats; mRNA levels rose 3.5-fold in CON rats, but only doubled in SUP rats



Figure 30. GFAP Expression 16 Days After Saline or KA Treatment in CON and SUP Rats. Comparison between CON (white bars) and SUP rats (grey bars) in GFAP protein levels (A) and mRNA levels (B) (mean ± SEM percent of control levels) in the intact hippocampus (open bars) and 16 days following KA-induced SE (hatched bars). GFAP protein levels were quantified using Western blot analysis (A). SE significantly increased GFAP protein and mRNA levels in both CON and SUP rats (ps < 0.05), but this increase was significantly attenuated in SUP rats. * statistically different from within-diet saline-treated group; # statistically different from KA-treated CON.

(Figure 30B, ps < 0.05). A similar, but not significant, pattern of change was observed for GFAP protein levels: a 3.1-fold increase following KA in CON rats, but only a 2.6-fold increase in SUP rats (Figure 30A, p = 0.07). Collectively, these data suggest that SE-induced upregulation in GFAP levels may be attenuated in SUP rats.

Growth factor expression in the intact and KA-lesioned hippocampus as a function of prenatal choline supplementation

The expression of growth factors is thought to be an important mediator of hippocampal neurogenesis (Cameron et al., 1998; Anderson et al., 2002; Lee et al., 2002a; Lee et al., 2002b) and some factors are known to change in response to SE (Gall and Isackson, 1989; Gall et al., 1991; Mudo et al., 1996; Schmidt-Kastner et al., 1996; Katoh-Semba et al., 1999; Shetty et al., 2004). We therefore quantified hippocampal protein levels (via ELISA) of the following growth factors known to influence neurogenesis: BDNF, IGF-1, FGF-2, NGF, and NT-3. Figure 31 shows the relative hippocampal protein levels for each growth factor in CON and SUP saline- and KAtreated rats 16 days post-treatment (protein levels expressed as percent of control levels because protein concentrations varied greatly between growth factors).

Because we were ultimately interested in the potential effect of prenatal choline availability on hippocampal growth factor content in baseline conditions and in response to SE, planned comparisons were conducted between saline-treated CON and salinetreated SUP rats, as well as between saline-treated and KA-treated rats within each diet condition. In adult rats that did not experience seizures, protein levels of BDNF (p <0.001), IGF-1 (p < 0.05), and NGF (p < 0.05) were significantly higher in prenatal SUP



Figure 31. Hippocampal Growth Factor Expression 16 Days After Saline or KA Treatment in CON and SUP Rats. Comparison between CON (white bars) and SUP (grey bars) rats in growth factor protein levels (mean ± SEM percent of control levels) in the intact hippocampus (open bars) and 16 days following KA-induced SE (hatched bars). Protein levels were quantified using ELISA. SE led to a significant increase in BDNF, IGF-1, FGF-2, NGF, and NT-3 protein levels in CON rats, but a significant increase in only BDNF and IGF-1 protein levels was noted for SUP rats. Baseline protein levels of BDNF, IGF-1, and NGF were significantly higher in SUP rats. * statistically different from within-diet saline-treated group; # statistically different from KA-treated CON; † statistically different from saline-treated CON.

rats than saline-treated CON rats. Although differences between saline-treated CON and SUP rats in protein levels of FGF-2 (p = 0.29) and NT-3 (p = 0.13) failed to reach statistical significance, the respective means are in a similar direction (Figure 31). Collectively, these results suggest that SUP rats have a higher baseline hippocampal protein content of several neurotrophic factors.

As expected, SE significantly increased protein levels of BDNF (p < 0.001), IGF-1 (p < 0.001), FGF-2 (p < 0.01), NGF (p < 0.01), and NT-3 (p < 0.05) in CON rats. In contrast, SUP rats exhibited a significant increase only in BDNF (p < 0.001) and IGF-1 (p < 0.001) in response to SE (Figure 31). There was a similar trend for a SE-induced increase in FGF-2 in SUP rats (p = 0.06). It is important to note that because protein levels of BDNF and IGF-1 were significantly elevated in saline-treated SUP rats, the increase in these neurotrophic factor levels following SE in supplemented rats (51% increase in BDNF; 59% increase in IGF-1) was not as large as it was in CON rats (BDNF: 72% increase; IGF-1: 81% increase). Together, these data suggest that prenatal choline supplementation either prevents or attenuates protein expression of some growth factors in response to SE.

Discussion

Experiment 3 demonstrated that increased availability of choline during early development produced a long-lasting change in the fetal brain that led to a dramatically reduced hippocampal response 16 days following KA-induced SE. Compared to control rats, adult offspring of dams that were fed a choline-enriched diet for just 1 week, mid-gestation, responded to SE with: 1) reduced hippocampal histopathology, 2) robust

protection against loss of GAD65 protein and mRNA expression, 3) marked attenuation of dentate cell proliferation, 4) dramatic reduction of GFAP mRNA expression (with a tendency for GFAP protein to be reduced as well), and 5) an altered pattern of growth factor expression. These data are particularly important because prior work has shown a savings in hippocampal-dependent learning and memory at a similar time point following excitotoxin-induced SE in prenatally choline-supplemented rats (Yang et al., 2000; Holmes et al., 2002), suggesting that increased choline availability to the fetus may alter neural development such that the adult brain is protected from SE-induced damage and cognitive impairment. While these data do not rule out the possibility that choline supplements may have neuroprotective actions at other periods, we know that choline has profound memory-enhancing effects if it is supplemented on ED12-17 or PD15-30, but not at other developmental or adult timeframes (Meck and Williams, 2003; Meck et al., 2008).

Consistent with previous findings (Holmes et al., 2002), prenatally choline supplemented rats (SUP) showed reduced cell loss and gliosis in CA1, CA3, and hilus compared to control-fed rats even though all rats experienced the same severity and duration of seizure activity. While widespread neurodegeneration and altered plasticity of the hippocampus is a notorious consequence of prolonged seizures (Niquet et al., 1994a; Fujikawa et al., 2000; Kotloski et al., 2002), GABAergic neurons are particularly vulnerable (Obenaus et al., 1993; Houser and Esclapez, 1996; Esclapez and Houser, 1999; Shetty and Turner, 2001). Thus, the finding that prenatal choline supplementation prevented the loss of hippocampal GAD65 protein and mRNA (though not GAD67

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response to SE) is particularly interesting. The current findings may represent a protection of GABAergic neurons following SE, although loss of functional inhibition may not be due to a loss of inhibitory neurons *per se* (Sloviter, 1987; Davenport et al., 1990; Esclapez and Houser, 1999). Rather, a loss in GAD65 expression may contribute to a disruption in GABAergic function, as mice deficient in GAD65 are susceptible to epileptic seizures (Kash et al., 1997). Alternatively, our SUP rats may show an early restorative upregulation in GAD65 production. Indeed, Houser and Esclapez (1996) have found that remaining GABAergic hippocampal neurons upregulate GAD65 mRNA 2-4 months, but not 3-14 days, following seizure-induced neuronal damage. Future studies are needed to determine if the savings seen in our SUP rats are in numbers of GABAergic neurons or in altered recovery of GAD expression or both.

SE produces a robust reactive astroglial response in the hippocampus, yet the current data reveal that seizure-induced increases in GFAP mRNA and dentate cell proliferation were significantly reduced in SUP rats. There was also a tendency for GFAP protein levels and hilar cell proliferation levels to be attenuated as well. SE-induced reactive astrogliosis plays a significant role in the aberrant synaptic remodeling of the hippocampus thought to underlie the development of epilepsy (Jorgensen et al., 1993; Niquet et al., 1994b; Represa et al., 1995). Reactive astrocytes that proliferate in response to seizures express several genes that promote neurite outgrowth (Represa et al., 1993; Niquet et al., 1994a; Niquet et al., 1994a; Niquet et al., 1994b; Stringer, 1996) and the increase in the expression of these genes coincides with both the emergence of mossy fiber sprouting and the period when both GFAP and hippocampal cell proliferation are

elevated (Khrestchatisky et al., 1995; Represa et al., 1995; Bengzon et al., 1997; Parent et al., 1997; Borges et al., 2006). An attenuation of seizure-induced astrogliosis in SUP rats may thus indicate a reduced progression of epilepsy following SE.

The current experiment did not detect any differences between diet groups in the number of immature neurons in the dentate gyrus following SE. Previous work has shown that dentate neuronal and astroglial proliferation following SE likely arises from independent progenitor pools: inhibiting hippocampal neurogenesis before SE does not prevent astrogliosis or mossy fiber sprouting (Parent et al., 1999). Thus, prenatal choline supplementation may have a preferential effect on just one type of proliferative hippocampal response following SE. Although the current experiment did not detect changes in the number of DCX+ dentate and hilar neurons following seizures as a function of prenatal choline supplementation, it remains to be determined whether prenatal choline supplementation alters specific properties (e.g., migration pattern, morphology, long-term survival, maturation, functionality) of newborn neurons following seizures that are hypothesized to contribute to the development of epilepsy (Scharfman, 2004; Scharfman and Gray, 2007).

In order to further investigate the possible mechanisms of prenatal cholineinduced neuroprotection, the expression of neurotrophic factors that are known to support hippocampal plasticity and that are regulated by seizure activity were examined. It is well established that 1 hour to 4 days after SE, hippocampal NGF and BDNF are transiently increased while NT-3 levels are downregulated (e.g., Gall et al., 1991; Rocamora et al., 1994; Marcinkiewicz et al., 1997; Shetty et al., 2004). Less is known about growth factor responses beyond one week after SE, but hippocampal NGF and NT-3 protein are still upregulated 45 days following SE while BDNF returns to baseline (Shetty et al., 2003). The current experiment adds to this timeline by demonstrating large increases in hippocampal BDNF, IGF-1, and FGF-2 protein 16 days post-SE in control-fed rats. Modest seizure-induced increases in hippocampal NGF and NT-3 were also observed. Thus, the hippocampal growth factor response to SE may be characterized by periods of elevation and/or reduction in levels that are specific for each factor. Levels of BDNF and IGF-1 protein were significantly elevated in both SUP and CON rats following KAtreatment, although the percent increase in these growth factors following SE compared to baseline levels was reduced in the SUP rats. These data may suggest that the response of these growth factors to SE is attenuated, or that some ceiling prevents further increases in the SUP rats. The latter hypothesis seems somewhat unlikely because we find that the NGF and NT-3 protein response to SE is completely attenuated in the KA-treated SUP rats compared to KA-treated CON rats, suggesting that prenatal choline supplementation reduces the dramatic elevation of several growth factors following SE. Previous work has shown that blocking NGF's actions prior to kindling both retards kindling and prevents mossy fiber sprouting (Rashid et al., 1995; Van der Zee et al., 1995); therefore, the attenuated NGF response to KA-induced in SUP rats may confer protection against mossy fiber sprouting. Whether the muted response of some growth factors to SE in our supplemented rats underlies the finding of reduced cell proliferation following SE remains to be determined.

What may be key to understanding the neuroprotection conferred by prenatal choline supplementation observed here, as well as the choline-induced protection against memory deficits following seizures reported previously (Yang et al., 2000; Holmes et al., 2002), is an altered hippocampal microenvironment in SUP rats prior to SE. SUP rats showed enhanced baseline levels of several growth/neurotrophic factors (BDNF, NGF, IGF-1) in the hippocampus, compared to CON rats. This finding extends our previous demonstrations of higher BDNF protein in 8-month old (Glenn et al., 2007) and NGF protein in 20- and 90-day old (Sandstrom et al., 2002) female offspring of cholinesupplemented dams. Infusions of BDNF or bFGF into the hippocampus prior to SE (Liu et al., 1993; Liu and Holmes, 1997; Reibel et al., 2000) as well as exercise (Setkowicz and Mazur, 2006), which stimulates BDNF, NGF, and IGF-1 (Gomez-Pinilla et al., 1998; Cotman and Berchtold, 2002), have been shown to ameliorate the progression of excitotoxin-induced SE. Prior exercise also mitigates learning and memory deficits shortly after KA-induced seizures (Gobbo and O'Mara, 2005). These data support the hypothesis that higher baseline growth factor levels in our SUP rats prior to SE may confer a neuroprotective hippocampal microenvironment that would make SUP rats more resilient to the deleterious effects of seizures.

Although the current experiment did not detect a significant increase in baseline dentate neurogenesis with prenatal choline supplementation in young adult males, Experiment 1 demonstrated higher baseline hippocampal neurogenesis/dentate cell proliferation in 7-9-month old SUP female rats compared to age-matched controls, which has also been similarly reported for 8-month old SUP female rats (Glenn et al., 2007), 16month old SUP male rats (Wong-Goodrich et al., 2008a), and 25-month old SUP male and female rats (Glenn et al., 2008b). The inability to detect differences in baseline neurogenesis in the present experiment may be due to a high baseline rate of neurogenesis in the 2-month old rat (Kuhn et al., 1996; Rao et al., 2006), which may mask any effects of prenatal choline.

Enhanced hippocampal plasticity likely plays a significant role in both the memory-enhancing effects of prenatal choline supplementation (Meck and Williams, 2003; McCann et al., 2006) and its neuroprotective actions. Much like our prenatal choline supplemented adult rats, juvenile rats that experience SE also show resistance to seizure-induced hippocampal cell loss, disinhibition, and mossy fiber sprouting (Sperber et al., 1991; Haas et al., 2001), and exhibit savings in spatial learning and memory (Stafstrom et al., 1993; Sarkisian et al., 1997) when compared to adult rats that experience SE. The retention of juvenile-like neuroplasticity and response to seizures into adulthood may contribute to prenatal choline supplementation's preservation of cognitive function in the face of a neural insult.

EXPERIMENT 4: PRENATAL CHOLINE DEFICIENCY DOES NOT ALTER HIPPOCAMPAL RESPONSE TO SEIZURES IN ADULTHOOD

Because prenatal choline supplementation is neuroprotective, as demonstrated in Experiment 3 and by others (e.g., Yang et al., 2000; Guo-Ross et al., 2002; Holmes et al., 2002; Guo-Ross et al., 2003), one hypothesis is that prenatal choline deficiency may enhance vulnerability to neural injury. However, little is known about the effects of prenatal choline deficiency on the adult brain's response to injury, and the few available studies have not provided much support in favor of this hypothesis. For example, while prenatal choline supplementation has been shown to protect against NMDA receptor antagonist-induced neurotoxicity (i.e., neuronal cell death) in the adult posterior cingulate and retrosplenial cortices, prenatal choline deficiency had no effect on the severity of toxicity-induced neuronal cell death in these neocortical regions compared to control-fed rats (Guo-Ross et al., 2003).

Similarly, another study reported that at two weeks after KA-induced SE, prenatally choline deficient adult rats exhibited similar levels of hippocampal cell loss as control-fed rats (Holmes et al., 2002). These researchers also examined spatial learning on a standard reference memory water maze task and found that prenatally choline deficient rats were just as impaired as control rats at 2 weeks after SE, despite KA-treated deficient rats having significantly lower levels of hippocampal choline acetyltransferase (ChAT), which presumably leads to a reduction in the availability of acetylcholine for synaptic transmission, compared to KA-treated control-fed rats (Holmes et al., 2002). Given that neurogenesis in the adult dentate gyrus is modulated by cholinergic transmission (Cooper-Kuhn et al., 2004; Kaneko et al., 2006), it is possible that decreased cholinergic neurotransmission in the hippocampus of prenatally choline deficient rats following seizures may alter both seizure-induced neurogenesis, and perhaps other factors that contribute to the hippocampal response to seizures. Thus, one aim of Experiment 4 was to examine dentate proliferation/neurogenesis and other indices of pathophysiology following KA-induced SE (as measured in Experiment 3) to assess whether prenatal choline deficiency alters the neuropathological response to seizures in the adult hippocampus.

Previous work from our laboratory has shown that compared to control-fed rats, prenatal choline supplemented and prenatal choline deficient rats do not upregulate dentate cell proliferation and neurogenesis in response to goal-directed spatial exploration on a radial arm maze (Glenn et al., 2007). However, Experiment 3 demonstrated that in response to seizures, prenatal choline supplemented rats exhibited a robust enhancement in dentate cell proliferation, though this seizure-induced increase was markedly attenuated compared to prenatally control-fed rats, as were several other markers of neuropathology. To determine whether the inability of prenatal choline *deficient* rats to upregulate in response to maze exploration also pertains to seizures, a second aim of Experiment 4 was to investigate whether prenatal choline deficiency alters the proliferative response observed in the dentate gyrus shortly following kainic acid-induced seizures. Dissociating the effects of prenatal choline deficiency and supplementation on reactive dentate proliferation/neurogenesis may provide evidence that the effects of

prenatal choline status on reactive neurogenesis may operate via different mechanisms depending upon the type of neurogenic stimuli.

Methods

Animals

Prenatal control (CON) and deficient (DEF) male rats were produced as described in Experiment 3. Briefly, dams were fed a control diet ad libitum (AIN76-A from Dyets, American Institute of Nutrition, ICN, Nutritional Biochemical, Cleveland, Ohio; 1.1 g/kg choline chloride substituted for choline bitartrate). On the evening of ED11 to the morning of ED18 (ED 12-17), pregnant dams were either given ad libitum access to a control diet (n = 29) or a diet deficient in choline (n = 15). Control and deficient dams were also given water sweetened with 50 mM saccharine because these dams were treated alongside choline supplemented dams. On the morning of ED18, all dams were returned to normal drinking water. There were no significant differences in the amount of water intake, food consumed, or body weights on ED11-18 between control and deficient dams (ps > 0.05; data not shown). After birth, offspring from the control and choline deficient dams were toe clipped for identification and then were selected randomly and cross-fostered to dams that consumed the control diet throughout pregnancy to yield 10 pups per litter (5 males and 5 females, with a equal representation of control, deficient, and supplemented offspring per litter). There were no significant differences between control and prenatally choline deficient litter size or pup birth weights (ps > 0.05). On postnatal day (P) 25, pups were weaned and pair-housed with a rat of the same sex and prenatal diet condition. All offspring were given ad libitum access to the control diet

through the duration of the study. Male offspring were used as subjects. All animal procedures were in compliance with the Institutional Animal Care and Use Committee of Duke University.

Induction of status epilepticus and bromodeoxyuridine injections

A timeline of experimental procedures is summarized in Figure 32. Status epilepticus (SE) induction procedures were as described in Experiment 3, with the exception that kainic acid (KA) was obtained from Tocris Bioscience (Ellisville, MO). KA was dissolved in 0.9% sterile saline (Sigma, St. Louis, MO). On P60, a group of adult male control offspring (CON, n = 12) and prenatally choline deficient offspring (DEF, n= 13) were injected with KA (2.5 mg/kg, i.p.) every hour to induce SE. A separate group of CON (n = 8) and DEF (n = 6) male offspring was similarly treated with hourly injections of 1 ml/kg saline. Although more DEF rats did not survive the KA treatment, mortality rates did not differ significantly between CON rats (4/12) and DEF rats (8/13), $\chi^2(1, N = 25) = 1.99$, p = 0.24. As in Experiment 3, five days after being treated with KA or saline, all rats were administered daily injections of 5-bromo-2-deoxyuridine (BrdU; 100mg/kg/day, i.p.; Sigma, St. Louis, MO) for 10 consecutive days to label dividing cells. All rats were then sacrificed 24 hours after the last injection at 16 days after KAinduced SE.



Figure 32. Experiment 4 Timeline of Experimental Procedures. 161

Tissue harvesting, cresyl violet staining, BrdU immunohistochemistry, doublecortin immunohistochemistry, quantification of BrdU- and DCX-labeled cells using unbiased stereology, ELISA for neurotrophic and growth factors, Western blot analysis for GAD65, GAD67, and GFAP, and Reverse transcriptase PCR

All procedures were performed as described in Experiment 3.

Data analyses

Numbers of BrdU-labeled and DCX-labeled cells estimated with the optical fractionator, the volumes of dentate gyrus that were estimated using Cavalleri's principle, and protein and mRNA levels (expressed as percent of control levels) were all subjected to a 2 (Diet: CON vs. DEF) × 2 (Seizure: saline vs. KA) between-subjects ANOVA. Of particular interest were Diet × Seizure interactions. Where appropriate, *a priori* pairwise comparisons were used to evaluate differences between group means. A significance level of 0.05 was set for all statistical tests. Values are reported in the text as means \pm SEM. Note that subjects contained within each experimental condition were randomly selected from different litters (*n* of 1/litter). Thus, necessary precautions were taken to be sure that the findings are not contaminated by a lack of within-litter variability.

Results

Seizure activity and hippocampal histopathology

All rats treated with KA reached SE and exhibited continuous Class III-V seizures for over 3 hours. Careful observation and analyses of behavioral activity of rats receiving KA revealed that the pattern of seizure activity (i.e., progression, number, and severity of seizures) was similar for both CON and DEF rats. There were no significant differences between CON and DEF rats in the number of Class III (CON = 1.00 ± 0.53 ; DEF = 1.40 ± 0.68), Class IV (CON = 4.37 ± 0.68 ; DEF = 5.00 ± 2.35), Class V (CON = 1.25 ± 0.62 ; DEF = 4.40 ± 1.81), or total number of motor seizures (CON = 6.62 ± 1.02 ; DEF = 10.80 \pm 4.63) observed within the first 1-hour period of observable seizure activity (all ps > 0.05). Rats treated with KA entered SE in a similar fashion to rats treated with KA in Experiment 3. After exhibiting their first motor seizure, the majority of both CON and DEF rats either entered a continuous Class III-V seizure state or exhibited ≥ 10 Class IV/V seizures in the second 1-hour period, and by the third hour all rats entered a continuous Class III-V seizure state. No differences in seizure severity or duration occurred beyond the 3-hr time window used to define SE. All rats gradually dropped out of SE within 2-3 hours of the last KA injection. During this time window, continuous seizures subsided, followed by the emergence of 1-3 discrete Class III/IV/V seizures, and all seizure activity abated by 5 hours after the last injection. There were no significant differences between CON and DEF rats in the total amount of KA needed to induce SE $(CON = 8.84 \pm 1.11 \text{ mg/kg}; DEF = 10.00 \pm 1.31 \text{ mg/kg})$, or in the latency to the first motor seizure (CON = 172.88 ± 23.01 min; DEF = 201.00 ± 19.16 min). The variability in dose and latency to the first motor seizure in our KA-treated rats is consistent with Experiment 3 and previous reports showing that the Sprague Dawley rats show a more variable convulsant response to KA than other rat strains (Golden et al., 1991; Golden et al., 1995). Thus, while the dose of KA needed to induce SE varied across rats, the SE produced was quite comparable between our CON and DEF rats, which is consistent with previous work using a single universal dose of KA to induce SE in prenatal choline control-fed versus deficient rats (Holmes et al., 2002).

KA-induced SE produced moderate to considerable cell loss, disruption of cellular architecture, and gliosis in areas CA1, CA3, and the hilus in both CON and DEF rats. The granule cell layer of the dentate gyrus remained relatively intact. There were no apparent differences in hippocampal lesion severity between KA-treated CON and DEF rats (Figure 33). As in Experiment 3, histopathology scores adapted from previous reports (Yang et al., 2000; Holmes et al., 2002), which were made by two independent observers blind to the diet treatment condition of each rat, were used to confirm that the KA treatment lead to similar patterns of hippocampal histopathology scores between CON and DEF rats. There were no significant differences in histopathology scores between KA-treated CON rats (2.13 ± 0.32) and DEF rats (2.00 ± 0.27) , which is consistent with a prior study showing statistically similar pathology scores between prenatal choline control and deficient rats at two weeks after KA-induced SE (Holmes et al., 2002).

Seizure-induced decreases in hippocampal GAD mRNA and protein are not modulated by prenatal choline deficiency

Hippocampal GAD65 and GAD67 mRNA and protein (via Western blot) levels in saline-treated and KA-treated CON and DEF rats at 16 days after treatment were quantified and these data, expressed as percent of control values, and are shown in Figure 34. Both CON and DEF rats exhibited a seizure-induced decrease in all GAD measures at 16 days after SE, which was confirmed by a main effect of Seizure for GAD65 protein, *F* (1, 23) = 10.01, p < 0.01, GAD67 mRNA, F(1, 22) = 5.86, p < 0.05, and GAD67 protein, F(1, 20) = 24.41, p < 0.001 (Figure 34). There was also a strong trend for a main effect of Seizure for GAD65 mRNA, F(1, 22) = 3.34, p = 0.08. These data are consistent with






Figure 34. Hippocampal GAD Expression 16 Days After Saline or KA Treatment in CON and DEF Rats. Comparison between CON (white bars) and DEF rats (grey bars) in GAD65 and GAD67 mRNA levels (*A*) and protein levels (*B*) (mean ± SEM percent of control levels) in the intact hippocampus (open bars) and 16 days following KA-induced SE (hatched bars). mRNA levels were quantified using RT-PCR and protein levels were quantified using Western blot analysis. An overall effect of Seizure was evident for GAD65 mRNA, GAD65 protein, and GAD67 protein (ps < 0.05) with a strong trend for GAD65 mRNA (p = 0.08) where KA-induced SE similarly reduced hippocampal GAD expression in CON and DEF rats. # main effect of Seizure at p < 0.05. * statistically different from within-diet saline-treated group at p < 0.05.

previous reports (Houser and Esclapez, 1996; Kobayashi and Buckmaster, 2003), as well as the seizure-induced reductions in hippocampal GAD65 and 67 content observed in KA-treated CON rats in Experiment 3. There were no effects of Diet or Diet × Seizure interactions for any GAD measure evaluated, and planned comparisons did not reveal any significant baseline differences in GAD levels between saline-treated CON and DEF rats. Thus, prenatal choline deficiency did not significantly alter hippocampal GAD levels or the reduction in hippocampal GAD65 and 67 mRNA and protein expression at 16 days after KA-induced SE.

Upregulation of cell proliferation and the number of immature neurons in the dentate gyrus shortly following seizures is not modulated by prenatal choline deficiency

To examine changes in dentate cell proliferation following KA-induced SE, cells that were immunopositive for the mitotic marker, BrdU, administered 5-15 days after SE were visualized and quantified using the optical fractionator method. Figure 35 shows photomicrographs of BrdU labeling in the hippocampus of representative sections from CON and DEF saline- and KA-treated rats. The pattern of BrdU labeling was similar to that detected in Experiment 3. The 10-day injection regimen of BrdU administration yielded a very high density of BrdU labeling in both saline- and KA-treated rats. BrdU+ cells were expressed throughout the rostral-caudal extent of the dentate gyrus and in both the suprapyramidal and infrapyramidal blades. BrdU+ cells were evident in the SGZ, GCL, and a vast majority of BrdU+ cells displayed morphological characteristics of normal non-pyknotic cells (e.g., round or oval nuclei that did not appear highly condensed). In KA-treated rats, a considerable portion of BrdU+ cells were also present in the hilus. KA-treated rats had a much greater density of BrdU labeling than salinetreated rats, but there were no apparent differences between prenatal CON and DEF rats in either condition. This observation was confirmed by quantification of BrdU+ cells in the SGZ-GCL and hilus of each rat using unbiased stereology and these data are presented in Figure 36. Analyses of the number of BrdU+ cells revealed a main effect of Seizure for both SGZ-GCL, F(1, 21) = 13.96, p = 0.001, and hilus, F(1, 21) = 9.42, p < 10000.01, but no effect of Diet or significant Diet × Seizure interaction for either region (Figure 36). Both CON and DEF rats exhibited a nearly threefold increase in the number of BrdU+ cells in the SGZ-GCL following seizures. Similar to Experiment 3, seizureinduced increases in hilar cell proliferation in CON and DEF rats in the current Experiment were much more robust. KA-treated CON rats showed an ~9-fold increase in hilar cell proliferation and KA-treated DEF rats showed an ~6-fold increase, though this difference was not significantly different (Figure 36). These data indicate that prenatal CON and DEF rats upregulated similar levels of dentate and hilar cell proliferation shortly after seizures.

To investigate whether prenatal choline deficiency altered the number of newborn neurons shortly after KA-induced seizures, cells that were immunopositive for the immature neuronal marker, DCX, were also quantified using unbiased stereology. Similar to Experiment 3, DCX+ neurons in the hippocampus of CON and DEF rats were visible along the SGZ and GCL (Figure 37). In KA-treated rats, DCX+ neurons were also present throughout the hilus region, and many DCX+ neurons in both KA-treated CON and KA-treated DEF rats appeared displaced and exhibited abnormal morphological features, such as horizontally oriented cell bodies and processes (Figure 37C, 37D). Unbiased stereological estimates of the number of DCX+ neurons in the SGZ-GCL (all rats) and in the hilus (KA-treated rats) were generated and these data are shown in Figure 38. KA-induced SE significantly increased the number of DCX+ neurons in the SGZ-GCL in both CON and DEF rats, F(1, 21) = 44.79, p < 0.001, but this seizure-induced upregulation of immature neurons was not modified by prenatal choline deficiency, as there was no effect of Diet or Diet × Seizure interaction (Figure 38). KA-induced SE elicited a near doubling of immature neurons in the SGZ-GCL in both CON and DEF rats (Figure 38). KA-treated CON and KA-treated DEF rats also showed similar levels of DCX+ neurons in the hilus region (p > 0.55); Figure 38). Thus, similar to the BrdU findings, prenatal choline deficiency does not appear to alter the seizure-induced upregulation in the number of immature neurons that are present in the dentate gyrus shortly after KA-induced SE.

To verify that the overall size of SGZ-GCL or hilus structure did not mask or account for any differences in numbers of new cells and immature neurons, the volumes of the SGZ-GCL and hilus regions in each rat were estimated based on the contours made during BrdU+ and DCX+ cell counting and using Cavalleri's principle (Mouton, 2002) and there were no significant differences in volumes between any treatment groups (Fs <

1).



Figure 35. BrdU Immunolabeling 16 Days After Saline or KA Treatment in CON and DEF rats. BrdU-immunopositive cells (i.e., newly generated cells) in the SGZ-GCL and hilus 16 days following saline treatment (A, CON; B, DEF) or KA-induced SE (C, CON; D, DEF). The number of BrdU-labeled cells significantly increased 16 days after SE for both diet groups. Photomicrographs in the left column were taken with a 10x objective and images in the right column were taken with a 40x objective. Bars indicate 50 µm. GCL, granule cell layer. SGZ, subgranular zone. H, hilus.



Figure 36. Quantification of BrdU+ Cells in the Dentate Gyrus 16 Days After Saline or KA Treatment in CON and DEF Rats. Mean (\pm SEM) numbers of BrdU+ cells detected in the SGZ-GCL and hilus of CON (white bars) and DEF (grey bars) rats 16 days following saline treatment (open bars) or KA-induced SE (hatched bars). SE significantly increased the number of BrdU+ cells in both CON and DEF rats (*ps* < 0.05). * statistically different from within-diet saline-treated group.



Figure 37. DCX Immunolabeling 16 Days After Saline or KA Treatment in CON and DEF Rats. DCX-immunopositive neurons (i.e., newly generated neurons) in the SGZ-GCL and hilus 16 days following saline treatment (A, CON; B, DEF) or KAinduced SE (C, CON; D, DEF). The number of DCX-labeled cells significantly increased 16 days after SE for both CON (A, C) and DEF (B, D) rats. Note that in KAtreated rats of both diet groups (C, D), DCX-positive neurons were aberrantly located in the hilus and exhibited abnormal morphological features, such as horizontally oriented cell bodies and processes (arrows). Photomicrographs in the left column were taken with a 10x objective and images in the right column were taken with a 40x objective. Bars indicate 50 µm. GCL, granule cell layer. SGZ, subgranular zone. H, hilus.



Figure 38. Quantification of DCX+ Cells in the Dentate Gyrus 16 Days After Saline or KA Treatment in CON and DEF Rats. Mean (\pm SEM) numbers of DCX+ cells detected in the SGZ-GCL and hilus of CON (white bars) and DEF (grey bars) rats 16 days following saline treatment (open bars) or KA-induced SE (hatched bars). KA-induced SE significantly increased the number of DCX+ cells in both CON and DEF rats (ps < 0.05). * statistically different from within-diet saline-treated group.

Prenatal choline deficiency does not alter SE-induced upregulation of hippocampal GFAP protein and mRNA expression

To examine whether prenatal choline deficiency alters reactive astrogliosis following seizures, hippocampal GFAP protein and mRNA levels were quantified in saline- and KA-treated CON and DEF rats at 16 days after treatment and these data, expressed as percent of control levels, are presented in Figure 39. Consistent with Experiment 3, KA-induced SE elicited a significant increase in hippocampal GFAP protein and mRNA levels in both CON and DEF rats. Analyses revealed a main effect of Seizure for both GFAP protein, F(1, 20) = 89.93, p < 0.001, and mRNA, F(1, 23) = 8.33, p < 0.01, but no effects of Diet or Diet × Seizure interactions, indicating that prenatal choline deficiency did not alter seizure-induced GFAP expression in the hippocampus.

Growth factor expression in the intact and KA-lesioned hippocampus as a function of prenatal choline deficiency

To examine whether prenatal choline deficiency alters seizure-induced changes in the hippocampal microenvironment, protein levels of various neurotrophic/growth factors that were measured in Experiment 3 (BDNF, IGF-1, FGF-2, NGF, and NT-3) were quantified (via ELISA) in the hippocampi of saline- and KA-treated CON and DEF rats at 16 days after treatment and the relative protein levels, expressed as percent of control values, are presented in Figure 40. Analyses revealed a main effect of Seizure for BDNF, F(1, 23) = 18.44, p < 0.001, and for FGF-2, F(1, 22) = 41.42, p < 0.001, but no effects of Diet or Diet × Seizure interactions for either growth factor. Both CON and DEF rats showed a large, significant increase in both BDNF and FGF-2 protein at 16 days after KA-induced SE (ps < 0.05; Figure 40). Planned comparisons revealed that saline-treated



Figure 39. Hippocampal GFAP Expression 16 Days After Saline or KA Treatment in CON and DEF Rats. Comparison between CON (white bars) and DEF rats (grey bars) in GFAP mRNA levels (*A*) and protein levels (*B*) (mean \pm SEM percent of control levels) in the intact hippocampus (open bars) and 16 days after KA-induced SE (hatched bars). mRNA levels were quantifed using RT-PCR and protein levels were quantified using Western blot analysis. SE significantly similarly increased GFAP mRNA and protein levels in both CON and DEF rats (ps < 0.05). # main effect of Seizure at p <0.05. * statistically different from within-diet saline-treated group. + different from within-diet saline-treated group at p = 0.07.



Figure 40. Hippocampal Growth Factor Expression 16 Days After Saline or KA Treatment in CON and DEF Rats. Comparison between CON (white bars) and DEF (grey bars) rats in growth factor protein levels (mean \pm SEM percent of control levels) in the intact hippocampus (open bars) and 16 days following KA-induced SE (hatched bars). Protein levels were quantified using ELISA. An overall effect of Seizure was evident for BDNF and FGF-2 protein (ps < 0.05) with a strong trend for IGF-1 protein (p= 0.06) where KA-induced SE similarly increased hippocampal growth factor expression in CON and DEF rats. Saline-treated DEF rats had significantly lower expression levels of IGF-1 and significantly higher expression levels of FGF-2 compared to saline-treated CON rats. * statistically different from within-diet saline-treated group at p < 0.05. + statistically different from saline-treated CON rats at p < 0.05.

DEF rats had significantly higher levels of hippocampal FGF-2 protein than salinetreated CON rats (p = 0.05; Figure 40), which meant that the increase in FGF-2 in KAtreated DEF rats was not as large (~3-fold) compared to KA-treated CON rats (~5-fold). There was a strong trend for a main effect of Seizure for IGF-1, F(1, 23) = 3.75, p = 0.06, suggesting that KA-induced SE likely increased hippocampal IGF-1 as well in both CON and DEF rats. Increases in BDNF, FGF-2, and IGF-1 at 16 days after KA-induced SE is consistent with the large increases in these growth factors observed in KA-treated CON rats in Experiment 3. There was also a main effect of Diet for IGF-1, F(1, 23) = 3.75, p =0.06, where DEF rats expressed lower overall levels of IGF-1 than CON rats, and planned comparisons revealed that saline-treated DEF rats had significantly lower levels of hippocampal IGF-1 protein than saline-treated CON rats (p = 0.01; Figure 40). There were no effects of Seizure, Diet, or Diet × Seizure interactions for levels of NGF or NT-3.

Discussion

Experiment 4 revealed that choline deficiency during ED 12-17 did not enhance vulnerability to KA-induced seizures in the adult offspring, as revealed by comparable levels of hippocampal histopathology, loss of hippocampal GAD mRNA and protein expression, increases in hippocampal GFAP and dentate cell proliferation and neurogenesis, and increases in several hippocampal growth factors at 16 days after excitotoxic injury between prenatally choline deficient and control-fed rats. These data are in concert with previous findings that also report no effect of prenatal choline deficiency on hippocampal histopathology or spatial learning deficits shortly following seizures (Holmes et al., 2002), or on neurotoxicity-induced neuronal cell death in the neocortex (Guo-Ross et al., 2003). Thus, while prenatal choline deficiency has been shown to compromise synaptic function and plasticity in the intact adult hippocampus (Pyapali et al., 1998; Jones et al., 1999; Montoya et al., 2000; Glenn et al., 2007), these findings suggest that prenatal choline deficiency does not appear to enhance the vulnerability of the hippocampus to excitotoxic seizures.

Perhaps somewhat surprising was that prenatally choline deficient rats had similar levels of dentate cell proliferation and neurogenesis in the adult hippocampus shortly after KA-induced seizures. This finding is of interest given that 1) prenatally choline deficient rats do not upregulate adult neurogenesis in the intact hippocampus in response to an enriching experience (Glenn et al., 2007), and 2) prenatal choline deficiency decreases hippocampal ChAT levels shortly after KA-induced SE, and adult neurogenesis is modulated by cholinergic activity (Cooper-Kuhn et al., 2004; Kaneko et al., 2006; Kotani et al., 2006; Kotani et al., 2008; Itou et al., in press). The dramatic seizure-induced increases in hippocampal growth factor expression observed in DEF rats in Experiment 4 may have been sufficient to both override the putative initial inability to upregulate dentate neurogenesis as well as compensate for losses in hippocampal cholinergic neurotransmission. A more elaborate discussion of this potential hypothesis is presented in the General Discussion. These data further suggest that prenatal choline deficiency's effects on reactive adult neurogenesis in the intact versus injured hippocampus may operate via different mechanisms.

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Experiment 4 did not detect differences in numbers of newly generated cells or immature neurons in the adult dentate gyrus between young adult saline-treated CON and DEF rats, which is consistent with previous work with 8-month-old female rats (Glenn et al., 2007). However, the results of the current experiment did reveal that prenatal choline deficiency reduced basal levels of hippocampal IGF-1 protein in young adult (~2-monthold) male rats, which is in concert with our previous study showing a similar reduction in hippocampal IGF-1 protein in prenatally choline deficient male rats at ~ 16 months of age (Wong-Goodrich et al., 2008a). Taken together, these findings suggest a persistent reduction in hippocampal IGF-1 levels by prenatal choline deficiency throughout life. While the precise role of IGF-1 in enrichment-induced hippocampal neurogenesis is not known, previous work has demonstrated that IGF-1 mediates exercise-induced enhancements in hippocampal neurogenesis and spatial memory (Trejo et al., 2001; Ding et al., 2006). Reductions in basal levels of IGF-1 in the intact hippocampus by prenatal choline deficiency may thus partially contribute to the inability to upregulate neurogenesis in response to environmental enrichment and/or physical activity, both of which were components in the maze exploration task in the previous study (Glenn et al., 2007).

Interestingly, basal levels of hippocampal FGF-2 were elevated in prenatally deficient rats compared to control-fed rats in the current experiment. Systemic or ICV administration of FGF-2 can increase adult hippocampal neurogenesis (Rai et al., 2007; Perez et al., 2009), and it has been suggested that FGF-2 makes an important contribution to the hippocampal trophic system that maintain levels of adult neurogenesis into old age

(Mudo et al., 2009). However, elevated FGF-2 can also inhibit adult hippocampal neurogenesis, but this inhibition may depend on the availability of other neurotrophic/growth factors, including IGF-1 (Chen et al., 2007). Future studies are needed to determine whether enhanced hippocampal FGF-2 (perhaps concomitant with reductions in IGF-1) conferred by prenatal choline deficiency translates into positive or negative effects on adult hippocampal neurogenesis.

Importantly, KA-induced SE produced in Experiment 4 yielded similar alterations in hippocampal GAD content, GFAP, dentate cell proliferation and neurogenesis, and growth factor expression in CON rats as that of CON rats in Experiment 3. There were, however, some small differences in the pattern of hippocampal growth factor expression following seizures. Overall, Experiment 4 revealed that KA-induced SE led to large increases in hippocampal BDNF, FGF-2, and a strong trend for IGF-1 at 16 days after excitotoxic injury. Large increases in these same growth factors were also observed in KA-treated CON rats in Experiment 3. In contrast to Experiment 3, Experiment 4 did not detect an effect of SE on hippocampal NGF or NT3 expression. However, the effects of KA-induced SE on NGF and NT3 in Experiment 3 were much smaller than the effects of SE on BDNF, FGF-2, and IGF-1, which may suggest that this effect is less reliable and may partially account for this disparity. In addition, due to the sudden lack of availability of Ocean Produce KA after the completion of Experiment 3, the KA for Experiment 4 was obtained from a different vendor (Tocris Bioscience), which may also have contributed to differences between experiments in the rats' response to the KA treatment.

EXPERIMENT 5: PRENATAL CHOLINE SUPPLEMENTATION AIDS LONG-TERM HIPPOCAMPAL RECOVERY FROM SEIZURES IN ADULTHOOD¹

The neuroprotective effects of prenatal choline supplementation on brain and behavior have been demonstrated shortly after excitotoxic injury. Prophylactic prenatal dietary choline supplementation protects young adult rats against seizure-induced spatial learning and memory retention deficits in the water maze observed 1-2 weeks after SE (Yang et al., 2000; Holmes et al., 2002). In support of these behavioral findings, Experiment 3 demonstrated that prenatal choline supplementation attenuated the neuropathological response to seizures just 16 days after status epilepticus. It is unknown, however, whether prenatal choline supplementation is a prophylactic treatment that can ameliorate the more long-term cognitive and neuropathological consequences of SE and/or whether the added plasticity conferred by prenatal choline supplementation might aid an injured hippocampus to engage in a cognitive task, which might further facilitate the long-term recovery from SE. Experiment 5 utilized the same kainic acid (KA) model of excitotoxic injury in adult rats to investigate whether prenatal choline supplementation and/or repeated cognitive testing in the water maze can alter the more long-term effects of SE on spatial learning and memory retention and on hippocampal neuropathology.

As previously stated, status epilepticus is a serious neurological condition that produces a myriad of degenerative and regenerative changes in the hippocampus, which are thought to contribute to the development of temporal lobe epilepsy in rodent models

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and in humans. Similar to short-term hippocampal changes following prolonged seizures, long-term hippocampal pathophysiology after SE includes considerable neurodegeneration (e.g., Cavazos et al., 1994; Haas et al., 2001; Gorter et al., 2003), yaminobutyric acid (GABA) system alterations (Morimoto et al., 2004; Sperk et al., 2004; Brooks-Kayal et al., 2009), reactive gliosis (e.g., Jorgensen et al., 1993; Niquet et al., 1994a; Stringer, 1996; Kang et al., 2006), aberrant mossy fiber innervation of the dentate gyrus (Sutula et al., 1988; Ben-Ari and Represa, 1990), altered growth factor levels (Shetty et al., 2003; Morimoto et al., 2004), and perturbed dentate cell proliferation and neurogenesis (Bengzon et al., 1997; Parent et al., 1997; Scharfman et al., 2000; Hattiangady et al., 2004). These plastic changes following SE are accompanied by cognitive deficits on hippocampal-dependent tasks, which are present both before and after the emergence of spontaneous recurrent motor seizures (Stafstrom et al., 1993; Liu et al., 1994; Sarkisian et al., 1997; Hort et al., 1999; Mikati et al., 2001; Kemppainen and Pitkanen, 2004; McKay and Persinger, 2004; Detour et al., 2005; Lin et al., 2009). As has been previously suggested (Prince et al., 2009), to develop prophylactic or rehabilitative strategies that might be applied either before or after brain injuries that lead to epilepsy, it is critical to understand the pathophysiological and cognitive processes to target.

Like prenatal choline supplementation, both environmental enrichment and physical exercise are also known to enhance hippocampal plasticity and cognitive function in the absence of injury (van Praag et al., 2000; Cotman et al., 2007), and may also be beneficial for the functional recovery from SE and temporal lobe epilepsy. Enrichment and/or exercise have been shown to reduce the frequency and severity of seizures, prevent neurodegeneration, and reverse deficits in synaptic plasticity (Dhanushkodi and Shetty, 2008; Hattiangady and Shetty, 2008b; Arida et al., 2009). Post-SE housing of immature rats in an enriched environment with access to a running wheel has been shown to ameliorate spatial learning and memory deficits in the water maze (Young et al., 1999; Faverjon et al., 2002; Rutten et al., 2002), although studies of the effects of experience on cognitive recovery after SE in adult animals are very limited. One recent study suggests that voluntary wheel running after SE in adult mice aids recovery of spatial learning and memory in the water maze (Sartori et al., 2009).

Experiment 5 had several goals. Place learning in the water maze offers an enriching learning experience that engages hippocampal networks (Jenkins et al., 2004; Kee et al., 2007), enhances the survival of newborn neurons, (Gould et al., 1999a), and has an exercise component. No study to date has examined whether repeated cognitive testing can rehabilitate spatial learning and memory and alter hippocampal recovery after SE. Second, while both prenatal choline supplementation and enrichment/exercise increase hippocampal plasticity and aid recovery from hippocampal injury, no study has directly compared the effects of these two manipulations on brain and cognitive function to determine if there are similar mechanisms underlying these effects. In particular, Experiment 5 focused on the effects of SE on a number of markers in the hippocampus that contribute to the neuropathological response to seizures, which were shown to be attenuated by prenatal choline supplementation shortly after SE (Experiment 3), and that may be potential therapeutic targets for the treatment of SE and epilepsy. These measures included hippocampal histopathology, glutamic acid decarboxylase (GAD) expression,

astrogliosis as measured by glial fibrillary acidic protein (GFAP) expression, dentate neurogenesis, and levels of brain derived neurotrophic factor (BDNF). Third, because prenatal choline supplementation enhances hippocampal plasticity in the intact adult hippocampus (Experiments 1 and 3; Sandstrom et al., 2002; Mellott et al., 2004; Glenn et al., 2007), of particular interest was whether prenatal choline supplementation would better enable the adult injured hippocampus to take advantage of the rehabilitative effects of cognitive enrichment after SE.

Methods

Animals

Prenatal control (CON) and supplemented (SUP) male rats were produced as described in Experiment 3. Briefly, 56 timed-pregnant dams were fed a control diet *ad libitum* (AIN76-A from Dyets, American Institute of Nutrition, ICN, Nutritional Biochemical, Cleveland, Ohio; 1.1 g/kg choline chloride substituted for choline bitartrate). On the evening of ED11 to the morning of ED18 (ED 12-17), pregnant dams were either given *ad libitum* access to a control diet (n = 29) or a choline supplemented diet (n = 17). Control dams were given the AIN76-A diet and water sweetened with 50 mM saccharine. Choline supplemented dams received the AIN76-A diet and water containing 25 mM choline chloride and sweetened with 50 mM saccharine. On the morning of ED18, all dams were returned to normal drinking water. There were no significant differences in the amount of water intake, food consumed, or body weights on ED11-18 between control and choline supplemented dams (ps > 0.05; data not shown). After birth, offspring from the control and choline supplemented dams were toe clipped for identification and then were selected randomly and cross-fostered to dams that consumed the control diet throughout pregnancy to yield 10 pups per litter (5 males and 5 females, half from different control dams and half from supplemented dams). There were no significant differences between control and prenatally choline supplemented litter size or pup birth weights (ps > 0.05). On postnatal day (P) 25, pups were weaned and pairhoused with a rat of the same sex and prenatal diet condition. All offspring were given *ad libitum* access to the control diet through the duration of the study. Male offspring were used as subjects. All animal procedures were in compliance with the Institutional Animal Care and Use Committee of Duke University.

Timeline of experimental procedures

The experimental design and timeline of procedures is presented in Figure 41. Adult male CON and prenatally choline supplemented SUP offspring were first trained in the water maze at approximately 56 days of age. Twenty-four hours after the last day of water maze training, CON and SUP rats were injected with saline or of kainic acid (KA) to induce status epilepticus (SE) and were given 5 days to recover. All rats were then injected with the cell division marker, bromodeoxyuridine (BrdU), every other day for 10 days. A subgroup of saline- and KA-treated rats were then given three additional water maze testing sessions at 3, 6.5, and 10 weeks post-SE, while the remaining rats remained pair-housed in their home cage, yielding the following experimental groups: salinetreated home cage rats (CON, n = 6; SUP, n = 5), saline-treated water maze rats (CON, n= 10; SUP, n = 9), KA-treated home cage rats (CON, n = 4; SUP, n = 6), and KA-treated water maze rats (CON, n = 7; SUP, n = 8). All rats were sacrificed at approximately 11 weeks after saline or KA treatment. The overall design was a 2 (prenatal diet: CON vs. SUP) × 2 (seizure: saline vs. KA) × 2 (experience: home cage vs. water maze) between-subjects design.



Figure 41. Experiment 5 Design and Timeline of Experimental Procedures. *Induction of status epilepticus*

SE induction and post-SE care procedures were as described in Experiment 3, with the exception that the KA was obtained from Tocris Bioscience (Ellisville, MO). KA was dissolved in 0.9% sterile saline (Sigma, St. Louis, MO). These procedures (see Experiment 3 Methods) were designed such that all KA-treated rats experienced similar duration and severity of seizure activity (see Results). Mortality rates did not differ significantly between CON and SUP rats, $\chi^2 (1, N = 57) = 0.12$, p = 0.74. Rats remained pair-housed throughout the duration of the experiment. All KA-treated rats were checked at least 3 times per week in their home cage for fighting and for spontaneous motor seizures. During these brief observations, spontaneous motor seizures were not observed in any rat in its home cage. However, 2 CON and 2 SUP rats that were treated with KA did exhibit a spontaneous motor seizure during the last spatial learning component of water maze testing (see water maze procedures), but it did not prevent the animals from completing the water maze task. In this case, these rats were immediately removed from

the maze, given a short rest period, followed by a "make-up" trial. Analysis of each data point collected indicated that these 4 animals performed similarly to other animals in their respective treatment group. It should be noted, however, that the occurrence of spontaneous seizures during and after SE induction was not systematically monitored and that others have used electroencephalographic (EEG) monitoring to show that abnormal spiking activity and nonconvulsive EEG seizures can occur after excitotoxic injury before the onset of the first motor seizure (Bertram and Cornett, 1993; Jessberger et al., 2007a; Raedt et al., 2009). It is therefore possible that the number of rats undergoing spontaneous seizure activity weeks following SE was underestimated in the current experiment.

Bromodeoxyuridine injections

Five days after being treated with KA or saline, all rats were administered a total of 5 daily injections of 5-bromo-2-deoxyuridine (BrdU; 100mg/kg/day, i.p.; Sigma, St. Louis, MO)—one injection every other day for 10 days—to label dividing cells. This injection regimen was designed to capture the period of time during which alterations in SE-induced dentate cell proliferation and hippocampal pathology as a result of prenatal choline supplementation were observed (see Experiment 3).

Water maze procedures

The maze apparatus was as described in Experiment 1. All rats received 2 days of pretraining to adapt to the room, experimenter, and water, and to learn that there was an escape platform in the pool. Rats were first placed on a hidden platform in the middle of the maze for ~ 10 s, then placed in the water near the hidden platform and gently guided

toward the platform. This was repeated 2-3 times per day. For all training trials, rats were placed in a random start location facing the wall of the pool and given 60 s to locate a hidden platform. If a rat did not find the platform by the end of 60 s, it was gently guided to the platform. Rats were allowed to sit on the platform for 15 s after climbing on to it. Following each trial, rats were removed from the water maze and dried with a towel. Performance on the task, including latency and pathlength to locate a hidden platform as well as swim speed, was measured and recorded using a computerized tracking system (HVS Image, Hampton, UK).

Prior to saline or KA treatment (pre-SE), all rats were given four consecutive days (6 training trials per day with an inter-trial interval of 5-8 min) of water maze training where rats were trained to navigate the maze to learn a single platform location. Each rat's total latency to locate the hidden platform (summed across 6 trials) was recorded for each day. This measure has been used previously to assess water maze performance before and after excitotoxin-induced SE (Yang et al., 2000; Holmes et al., 2002). Following saline or KA treatment, a subgroup of CON and SUP rats were given three additional water maze testing sessions at 3, 6.5, and 10 weeks post-SE that included both a spatial learning and a spatial memory retention component. Each post-SE testing session was 5 days long and was separated by 21 days. On the first day of each session, a single spatial memory retention test was administered. Rats were required to locate the hidden platform that was placed in the same spatial location learned 3 weeks prior to the retention test. Latency and pathlength to locate the previously learned platform was recorded for each rat. In the first post-SE session (3 weeks post-SE), rats were tested for

their ability to remember the platform location they learned during the pre-SE training period (prior to saline or KA treatment). During the remaining 4 days of each session, rats were administered 6 training trials per day where they were required to learn a new platform location. Total latency and pathlength (summed across the 6 training trials) was recorded for each rat across each day.

Tissue harvesting, reverse transcriptase PCR, Western blot analysis for GAD65, GAD67, and GFAP, and BDNF ELISA

Procedures were as described in Experiment 3. At approximately 11 weeks after saline or KA treatment (24 hours after the last day of water maze training), rats were given an overdose of a ketamine/xylazine cocktail, decapitated, and brains were rapidly removed.

BrdU, DCX, and NeuN immunohistochemistry

Immunohistochemical procedures for BrdU- and DCX-labeling were as described in Experiment 1 and Experiment 3, respectively. Immunohistochemical procedures for NeuN-labeling were based on previous methods (Hattiangady et al., 2004). Free-floating sections were rinsed with tris-buffered saline (TBS: pH 7.3) followed by 30 min in 50% methanol and 30 min in 3% hydrogen peroxide in TBS at room temperature (RT) to reduce nonspecific staining, and then rinsed in TBS. Sections were incubated in 0.1% Triton X-100 (TTX; Sigma) and 3% normal horse serum (Vector Laboratories, Burlingame, CA) in TBS for 30 min at RT, and then incubated with the primary antibody (monoclonal mouse anti-NeuN, 1:500, Millipore) for 48 hours at 4 °C. Following this, sections were rinsed with TBS and incubated with the secondary antibody (biotinylated horse anti-mouse, 1:200; Vector Laboratories) for 2 hours at RT. Sections were then rinsed in TBS, incubated in an avidin-biotinylated peroxidase complex (ABC, Vector Laboratories) for 1 hour, rinsed again in TBS, and treated for peroxidase detection with diaminobenzidine (Vector Laboratories) for 4 min. Stained sections were mounted on gelatin-coated slides, dehydrated, and coverslipped.

Dual immunofluorescence for BrdU and NeuN

Double immunofluorescent labeling procedures were adapted from previous reports (Mirescu et al., 2006; Segi-Nishida et al., 2008). Four to five free-floating sections were rinsed with phosphate buffered saline (PBS) followed by 30 min in 50% methanol at room temperature to quench unperfused blood vessels and reduce nonspecific staining, and 30 min in 0.1% sodium tetrahydridoborate to reduce autofluorescence. After rinsing again in PBS, sections were treated for 10 min in 0.9% saline, followed by 30 min in 2N HCl at 37°C. Sections were rinsed in PBS, incubated in 0.3% Triton X-100 (TTX; Sigma) and 5% normal donkey serum (Jackson Immuno) in PBS for 30 min at room temperature, and then incubated with a primary antibody cocktail (polyclonal sheep anti-BrdU, 1:100, Abcam Inc.; monoclonal mouse anti-NeuN, 1:50, Millipore) for 48 hours at room temperature. Following this, sections were rinsed with PBS and incubated with the fluorescent secondary antibody Alexa Fluor 488 anti-mouse, 1:200 (Molecular Probes) for 24 hours at 4 °C. Sections were then rinsed in PBS, incubated in a biotinylated anti-sheep secondary antibody, 1:500 (Jackson Immuno) for 2 hours, rinsed in PBS, and then incubated in a streptavidin-conjugated Alexa Fluor 555, 1:500 (Molecular Probes) for 2 hours. Stained sections were then rinsed in PBS, mounted

on gelatin-coated slides with Vectashield anti-fading mounting medium (Vector Labs), coverslipped, and stored in the dark at 4 °C.

Quantification of BrdU+, DCX+, and NeuN+ cells using unbiased stereology

BrdU-, DCX-, and NeuN-labeled cells in the dentate gyrus were counted using a modified optical fractionator method (West, 1993; West, 1999; Mouton, 2002). For counting of BrdU+ and DCX+ cells, we sampled every fifth section through the rostralcaudal extent of the dentate gyrus in two sampling regions: one region included the subgranular zone (SGZ), which was designated as an approximately 2-cell thick zone between the inner rim of the granule cell layer (GCL), and the hilus, and the GCL that encompassed the suprapyramidal and infrapyramidal granule cell blades, and the other region included the hilus, which did not include the CA3c region. StereoInvestigator (Microbrightfield Inc., Williston, VT) was used to sample systematically throughout each SGZ-GCL region of all rats and count numbers of immunopositive cells, using an 80 x 80 μ m counting frame and ~10-60 sites per section in 8 sections that captured the rostralcaudal extent of the dentate gyrus. Optical fractionator estimates were multiplied by 2 to account for both hemispheres. For the hilus region, there was sporadic labeling of BrdU+ cells in all saline-treated rats, and sporadic labeling (or no labeling) of DCX+ cells in all treatment groups. In these cases, the hilus region was sampled exhaustively for immunopositive cells (as such, total counts were multiplied by 5 and then by 2 to account for both hemispheres). However, due to the high density of BrdU+ cells in the hilus of KA-treated rats, we systematically sampled using the optical fractionator throughout the hilus region in this case. These parameters ensured adequate sampling of BrdU+ and

DCX+ immunolabeled cells throughout the SGZ-GCL and hilus. The same parameters were used for counting NeuN+ cells with the exception of using 5 representative sections that captured the rostral-caudal extent of the hippocampus and a smaller counting frame $(15 \times 15 \mu m)$ due to the very high density of NeuN+ neurons in the GCL. For analysis, we set an optical dissector height of 20 µm with a 2-µm guard zone to avoid oversampling and counted stained cells in each frame using a 40x objective lens. Gundersen coefficient of error values were ≤ 0.10 for all optical fractionator estimates for each animal, with a range of 0.04 to 0.10. For each section examined, the area of the dentate gyrus was calculated by the StereoInvestigator software and was based on the boundaries of the contour tracings. Volume estimates were obtained by multiplying the section area estimates with the spacing between sampled areas. Spacing was derived by multiplying the measured, post-histology thickness of each sample by the number of sections examined, which was constant for all sections for all rats ($\sim 50\%$ shrinkage). Estimates of total hippocampal volume and of granule cell density were generated for all KA-treated rats and a subgroup of saline-treated rats (n = 4 per treatment group). For total hippocampal volume, contours were made around the entire half hippocampus in each coronal section of a single one-in-five series (stained with cresyl violet) throughout the rostral-caudal extent of the hippocampus. Total hippocampal volume and GCL volume estimates were generated according to Cavalleri's principle (Mouton, 2002) and were multiplied by 2 to account for both hemispheres. Granule cell density estimates were generated by dividing the optical fractionator estimate of the total number of NeuN+ neurons in the GCL by the volume estimate of the GCL.

Phenotypic analysis of BrdU+ cells using confocal microscopy

Procedures used to phenotype and quantify BrdU+ cells co-labeled with NeuN were adapted from previous reports (Mirescu et al., 2006; Hattiangady and Shetty, 2008a; Segi-Nishida et al., 2008). For dual immunofluorescence analyses, 25-50 BrdU+ cells in the SGZ-GCL of saline- and KA-treated rats (n = 4 per group) and all BrdU+ cells that met our selection criteria (see below) in the hilus of KA-treated rat (n = 4 per group) were analyzed for co-labeling of NeuN using a Zeiss Axio Observer inverted confocal laserscanning microscope equipped with LSM 510 software. Rat brains selected for confocal analysis (n = 4 per group) yielded treatment group means of BrdU+ cell counts that were comparable to overall treatment group mean BrdU+ cell counts. The hilus region of saline-treated rats was not analyzed due to a very small number of observed hilar BrdU+ cells. Due to the increased density of BrdU immunofluorescence of varying intensities in the hilus of KA-treated rats, immunopositive cells selected for analysis included BrdU+ cells that exhibited strong immunofluorescence (i.e., that were distinctly brighter than the background and that could be detected with a pinhole $\leq 1 \mu m$) with a more pronounced nuclei for phenotypic analyses. Cells that did not match a neural/glial cell morphology were excluded. For example, putative BrdU+ endothelial cells that were dispersed throughout the entire hilar region and had a nuclear morphology and placement consistent with endothelial cells were excluded (Scott et al., 2000; Hellsten et al., 2004). These cells had very weak immunofluorescence, were smaller and had more elongated nuclei, and did not express NeuN. BrdU+ cells were individually examined for the coexpression of NeuN using z-sectioning at 1 μ m intervals at a 40x objective. Percentages of BrdU+ cells

that coexpressed NeuN were individually calculated for each rat analyzed (n = 4 per group) and then multiplied by the total number BrdU+ cells for each rat to yield an estimated number of new neurons.

Data analyses

Data were analyzed using ANOVAs, post-hoc tests, and *a priori* comparisons to evaluate differences between specific group means where appropriate. A significance level of 0.05 was set for all statistical tests. Values are reported in the text as means \pm standard error of the mean (SEM). Analyses of water maze performance are presented first for pre-SE spatial learning performance, followed by post-SE spatial learning performance, and then post-SE spatial memory retention performance. Analyses of all brain measures were organized to adequately address three main points of focus: 1) the long-term effects of KA-induced SE, 2) how prenatal choline supplementation modulates these effects, and 3) how the experience of additional water maze training following SE modulates the effects of SE and/or interacts with prenatal choline availability. Thus, for brain measures, the 2 (Diet) \times 2 (Seizure) \times 2 (Experience) design was decomposed into separate 2 (Seizure) \times 2 (Experience) ANOVAs for CON vs. SUP rats such that the effects of SE and water maze experience were evaluated with respect to each withindiet's (CON or SUP) own baseline (i.e., saline-treated home cage) condition. Results are first presented for CON rats, followed by SUP rats. Specific planned comparisons between CON and SUP rats are highlighted where appropriate. Note that subjects in each experimental condition were randomly selected from different litters (n of 1/litter). Thus,

the necessary precautions were taken to be sure that the findings were not contaminated by a lack of within litter variability.

Results

Induction of status epilepticus

All rats treated with KA reached SE and exhibited continuous Class III-V seizures for over 3 hours. Careful observation and analyses of behavioral activity of rats receiving KA revealed that the pattern of seizure activity (i.e., progression, number, and severity of seizures) was similar for both CON and SUP rats. There were no significant differences between CON and SUP rats in the number of Class III (CON = 0.82 ± 0.44 ; SUP = 0.86 \pm 0.27), Class IV (CON = 5.64 \pm 1.19; SUP = 4.14 \pm 0.88), Class V (CON = 4.55 \pm 1.61; SUP = 4.77 ± 1.02), or total number of motor seizures (CON = 11.00 ± 2.58 ; SUP = 9.43 \pm 2.01) observed within the first 1-hour period of observable seizure activity (all ps > 0.05). After exhibiting their first motor seizure, the majority of both CON and SUP rats either entered a continuous Class III-V seizure state or exhibited ≥ 10 Class IV/V seizures in the second 1-hr period, and by the third hour all rats entered a continuous Class III-V seizure state. No differences in seizure severity or duration occurred beyond the 3-hr time window used to define SE. All rats gradually dropped out of SE within 2-3 hours of the last KA injection. During this time window, continuous seizures subsided, followed by the emergence of 1-3 discrete Class III/IV/V seizures, and all seizure activity abated by 5 hours after the last injection. There were no significant differences between CON and SUP rats in the total amount of KA needed to induce SE (CON = 13.77 ± 1.44 mg/kg; $SUP = 13.13 \pm 0.64 \text{ mg/kg}$) or in the latency to the first motor seizure (CON = 270.4 ±

29.5 min; SUP = 252.3 ± 12.1 min). The variability in dose and latency to the first motor seizure in our KA-treated rats is consistent with Experiments 3 and 4, and other reports showing that the Sprague Dawley rats show a more variable convulsant response to KA than other rat strains (Golden et al., 1991; Golden et al., 1995). Thus, while the dose of KA needed to induce SE varied across rats, the SE produced was comparable between CON and SUP rats. All KA-treated rats were then pseudo-randomly assigned to either a home cage or water maze post-SE condition to ensure there were no significant differences in any of the above seizure measures between CON and SUP rats in either home cage or water maze post-SE condition (all ps > 0.05; data not shown).

Water maze performance

Prior to saline or KA injections, all rats were trained in the water maze for 4 days. During pre-SE training, all CON and SUP rats learned the platform location, as revealed by a decreased latency to locate the hidden platform over four days of training (Figure 42A). A two-way repeated measures ANOVA revealed a main effect of day, F(3, 162) =76.54, p < 0.001) with significant differences between all days except days 3 and 4 (ps <0.001), but no effect of prenatal diet (F < 1). Analyses of pathlength data revealed the same pattern of findings (data not presented) and there were no significant group differences in overall swim speed (CON = 0.23 ± 0.01 m/s; SUP = 0.24 ± 0.01 m/s; p =0.51), confirming that both diet groups did, in fact, learn the platform location with the same proficiency.

To assess the loss of spatial learning ability following SE and recovery over the next 10 weeks, rats were trained to learn a novel platform location in the water maze over

three separate 4-day sessions at 3, 6.5, and 10 weeks after SE. KA-treated rats had significantly higher escape latencies than saline-treated rats at all post-SE sessions (ps < 10.01), confirming that KA-induced SE elicited significant deficits in spatial learning (Figure 42B). To examine the extent of recovery in KA-treated CON versus, SUP rats, separate 2 (Diet) \times 4 (Day of Training) ANOVAs were performed for saline- and KAtreated rats for each session and these data are also presented in Figure 42B. Consistent with our predictions, prenatal choline supplementation did not affect spatial learning in saline-treated rats (Figure 42B). Spatial learning on this reference memory water maze task is not difficult for an intact rat, likely because it does not impose many cognitive demands, which is when prenatal choline supplementation memory enhancing effects are observed (Meck and Williams, 1999; Meck and Williams, 2003; Wong-Goodrich et al., 2008a). Analyses of KA-treated rats revealed a significant main effect of day for all three sessions (all ps < 0.05), indicating that both KA-treated CON and KA-treated SUP rats learned the new platform location across four days of training and suggesting some cognitive recovery of spatial learning. However, analyses also revealed a significant main effect of Diet at 10 weeks post-SE where KA-treated SUP rats had lower latencies than KA-treated CON rats, F(1, 13) = 4.61, p = 0.05 (Figure 42B). There was a similar trend at 3 weeks post-SE, F(1, 14) = 2.74, p = 0.12, and at 6.5 weeks post-SE, F(1, 14) = 3.64, p = 0.07. These data revealed that SUP rats showed an attenuated spatial learning deficit following KA-induced SE; KA-treated SUP rats appeared to locate the platform with more efficiency than the KA-treated CON rats.

To examine recovery of long-term memory retention, rats were tested at 3, 6.5 and 10 weeks post SE for their memory of a platform location learned 3 weeks prior to the test. Latency to locate the platform was recorded across each session and analyzed using separate repeated measures ANOVA for each treatment group to assess performance over time post-KA or -saline treatment. There was a significant effect of Session for both saline-treated CON rats, F(2, 18) = 8.41, p < 0.01, and saline-treated SUP rats, F(2, 16) = 15.73, p < 0.001. Both groups demonstrated a significant decrease in latency from the 3-week to 6.5-week and 10 week post-SE sessions, indicating an improvement in spatial memory retention by the second session in saline-treated rats (ps < 0.05; Figure 42C). Analyses did not reveal an effect of Session for KA-treated CON rats, indicating a lack of improvement of memory retention across post-SE sessions. Planned comparisons also revealed that KA-treated CON rats had significantly higher escape latencies than saline-treated CON and SUP rats at all three post-SE sessions (ps < 10.05), confirming a persistent spatial memory deficit in KA-treated CON rats. Similar to saline-treated rats, there was also a significant effect of Session for KA-treated SUP rats, F(2, 14) = 9.68, p < 0.01, where KA-treated SUP rats exhibited a progressive decrease in escape latency with each post-SE water maze session (Figure 42C). While KA-treated SUP rats had significantly higher latencies than saline-treated CON and SUP rats at 3 and 6.5 weeks post-SE (p < 0.05), KA-treated SUP rats' memory retention was remarkably comparable to that of saline-treated CON and SUP rats by 10 weeks post-SE (Figure 42C), suggesting complete recovery of spatial memory retention.



Figure 42. Water Maze Performance. (*A*) During pre-SE training, all CON (grey) and and SUP (black) rats learned to locate the hidden platform with similar decreasing total latencies over 4 days of training. (*B*) Compared to saline-treated rats (solid lines), KA-treated (dashed lines) rats had significant impairments in spatial learning (higher total latencies) across 4 days during each post-SE session, which was attenuated in KA-treated SUP rats. (*C*) SE also impaired spatial memory over a 3-week retention interval, but KA-treated SUP rats showed a complete recovery of spatial memory retention by 10 weeks post-SE. * significantly different from all other groups at p < 0.05.

Analyses of pathlength data revealed a similar pattern of findings for spatial learning and memory retention testing (data not presented). In addition, group differences in latency to find the platform during spatial learning and memory retention tests were not due to differences in mean swim speeds (saline-treated CON = 0.26 ± 0.01 m/s; saline-treated SUP = 0.25 ± 0.02 m/s; KA-treated CON = 0.26 m/s; KA-treated SUP = 0.26 ± 0.02 m/s; ps = n.s.).

Hippocampal lesion

Varying degrees of histopathology throughout the rostral-caudal extent of the hippocampi was observed in all KA-treated rats. KA-induced SE led to moderate to considerable cell loss and disruption of cellular architecture in CA1, CA3, and the hilus of both CON and SUP rats, which can be seen in Figure 43. In contrast, there was mild to no cell loss in the GCL of the dentate gyrus, which is consistent with previous reports using the KA model of SE (Covolan et al., 2000). Total hippocampal volume estimates were used to quantify hippocampal cell loss at approximately 11 weeks after initial injury. Separate analyses of total hippocampal volume estimates for CON rats (salinetreated home cage = 69.59 mm³ \pm 5.03; KA-treated home cage = 54.38 mm³ \pm 16.73; saline-treated water maze = 79.15 mm³ \pm 12.91; KA-treated water maze = 48.29 mm³ \pm 41.54) and SUP rats (saline-treated home cage = 66.79 mm³ \pm 5.03; KA-treated home cage = 52.02 mm³ \pm 13.52; saline-treated water maze = 74.21 mm³ \pm 11.19; KA-treated water maze = 55.91 mm³ \pm 7.13) revealed a significant main effect of Seizure for CON rats, F(1,15) = 6.68, p < 0.05, and a strong trend for a main effect of Seizure for SUP rats, F(1,18) = 3.97, p = 0.06. KA-treated CON rats had a 31% overall decrease in



Figure 43. Hippocampal Histopathology of CON and SUP Rats 11 Weeks After KA-Induced SE. Representative sections from the dorsal hippocampus of CON (*B*) and SUP (*C*) rats. Panel A depicts an intact hippocampus from a saline-treated CON rat. Saline-treated SUP rats also did not show any lesions (histology data not shown). Note significant cell loss (arrows) in CA1, CA3, and hilus regions while the dentate gyrus granule cell layer remained relatively intact. Patterns of lesion severity were similar across all KA-treated rats. Photomicrographs in each set were taken with a 4x objective. Bars indicate 250 µm. DG, dentate gyrus. H, hilus.
hippocampal volume compared to saline-treated CON rats, while KA-treated SUP rats had a 23% overall decrease. While SE reduced total hippocampal volume in all rats, there were no significant differences in hippocampal volume between any group of KA-treated rats (all ps > 0.05), suggesting that the KA treatment in the current experiment produced similar levels of hippocampal cell loss across all treatment groups weeks after excitotoxic injury.

Hippocampal GAD65 and 67 mRNA and protein

Disruption of hippocampal GABAergic function is a robust consequence of seizures (Obenaus et al., 1993; Morimoto et al., 2004; Brooks-Kayal et al., 2009). In Experiment 3, prenatal choline supplementation prevented the decrease in hippocampal GAD65 mRNA and protein expression observed 16 days after SE (Wong-Goodrich et al., 2008b). To investigate whether this protection against the loss of GABAergic function in the hippocampus persists for 11 weeks after SE, hippocampal mRNA and protein levels of GAD65 and 67, enzymes important for local GABA synthesis at synaptic (GAD65) and cytoplasmic (GAD67) sites (Erlander and Tobin, 1991; Esclapez et al., 1994) and for the packaging and release of GABA (Namchuk et al., 1997; Tian et al., 1999), were measured.

GAD65 and 67 mRNA and protein levels were expressed as percent of control values and were subjected to separate 2-way ANOVAs for CON and SUP rats with Seizure (saline vs. KA) and Experience (home cage vs. water maze) as between-subjects factors. Higher within-group variability is typically observed for KA-treated groups, which was also observed at 16 days after SE in Experiment 3 (Wong-Goodrich et al.,

2008b) and Experiment 4. Analyses revealed that CON rats were significantly affected by KA treatment and/or post-SE experience on all GAD measures quantified. Interestingly, two distinct patterns emerged for mRNA versus protein for both GAD65 and 67 in CON rats (see Figure 44). For GAD 65 mRNA, there was a reduction (~30%) in GAD65 mRNA in home cage CON rats following SE, with a trend toward recovery from the SEinduced loss of GAD65 mRNA in water maze trained rats (p = 0.10). As such, analyses revealed a significant main effect of Experience, F(1, 23) = 7.95, p = 0.01, where water maze trained rats showed elevated levels of GAD65 mRNA compared to home cage rats. There was no significant main effect of Seizure or Seizure × Experience interaction (Figure 44A). For GAD67 mRNA, there were no main effects of Seizure or Experience. However, a significant Seizure × Experience interaction, F(1, 23) = 5.57, p < 0.05, confirmed that similar to the pattern of GAD65 mRNA in CON rats, KA-induced SE led to a significant 43% decrease in GAD67 mRNA levels in home cage CON rats (p < 0.05), but did not affect GAD67 mRNA levels in water maze CON rats (Figure 44B). Together these analyses revealed that exposure to water maze training following KA-induced SE appears to rescue the decline in GAD mRNA in CON rats. Analyses of both GAD65 and 67 protein levels in CON rats revealed a significant main effect of Seizure (GAD65: F(1, 1)21) = 5.39, p < 0.05; GAD67: F(1, 21) = 8.36, p < 0.01), but no main effect of Experience or any Seizure × Experience interaction. In contrast to mRNA levels, KAinduced SE appeared to decrease GAD65 and 67 protein levels in CON rats regardless of post-SE experience (Figure 44C, 44D).





In contrast to CON rats, there was little effect of SE on hippocampal GAD levels in SUP rats. GAD65 and 67 mRNA levels and GAD65 protein levels in SUP rats were not affected by KA-induced SE in either home cage or water maze rats (Figure 44). Compared saline-treated home cage SUP rats, there was a 20% reduction in GAD67 protein levels in KA-treated home cage SUP rats (p < 0.05), but this reduction was not evident in KA-treated water maze SUP rats (Figure 44D). However, KA-treated home cage SUP rats and KA-treated water maze SUP rats were not significantly different.

Hippocampal GFAP mRNA and protein

Reactive astrogliosis (proliferative and hypertrophic astrocytes) in the hippocampus, as measured by increased levels of GFAP, is also a robust consequence of SE that has been shown to persist for weeks to months following excitotoxic injury (Jorgensen et al., 1993; Niquet et al., 1994a; Aronica et al., 2000; Shapiro et al., 2008). Experiment 3 demonstrated that prenatal choline supplementation attenuates this seizure-induced increase in hippocampal mRNA and protein levels at 16 days following KA-induced SE (Wong-Goodrich et al., 2008b). To examine whether this attenuation persists beyond 16 days after seizures, mRNA and protein levels of hippocampal GFAP at 11 weeks post-SE were quantified. GFAP mRNA and protein levels were expressed as percent of control levels and were subjected to separate 2-way ANOVAs for CON and SUP rats with Seizure and Experience as between-subjects factors. For CON rats, analyses revealed a main effect of Seizure for GFAP mRNA, F(1, 23) = 21.52, p < 0.001, and protein, F(1, 21) = 183.88, p < 0.001. There was also a main effect of Experience, F(1, 21) = 19.69, p < 0.001, and a Seizure × Experience interaction, F(1, 21) = 20.78, p < 0.001.

0.001, for GFAP protein in CON rats. While KA-induced SE led to a similar increase in hippocampal GFAP mRNA in KA-treated CON home cage rats and water maze rats, the SE-induced increase in hippocampal GFAP protein levels was significantly reduced in KA-treated CON rats that received additional water maze training after SE (p < 0.01; Figure 45). These data revealed that elevated levels of hippocampal GFAP mRNA and protein persist for at least 11 weeks after KA-induced SE, but that elevated levels of GFAP protein are attenuated with water maze training.

For SUP rats, analyses revealed a main effect of Seizure for both GFAP mRNA, F(1, 24) = 25.62, p < 0.001, and protein levels, F(1, 24) = 37.87, p < 0.001. There was also a significant effect of Experience, F(1, 24) = 13.98, p < 0.01, and Seizure \times Experience interaction, F(1, 24) = 5.36, p < 0.05, for GFAP mRNA. Unexpectedly, the increase in hippocampal GFAP mRNA levels was significantly higher for KA-treated SUP rats that received additional water maze training (p < 0.01; Figure 45A). Hippocampal GFAP protein levels, however, were increased similarly for KA-treated home cage and water maze SUP rats (Figure 45B). Planned comparisons revealed that the seizure-induced increase in hippocampal GFAP mRNA and protein levels for KA-treated SUP rats was attenuated in comparison to KA-treated CON rats: home cage CON rats showed a 125% and 153% increase in GFAP mRNA and protein, respectively, whereas home cage SUP rats showed only a 46% and 56% increase (ps < 0.05; Figure 45). These findings reveal that prenatal choline supplementation leads to a remarkable long-term attenuation in SE-induced increases in GFAP mRNA and protein levels that persists for 11 weeks following KA-induced SE.



Figure 45. Hippocampal GFAP Expression 11 Weeks After Saline or KA Treatment in CON and SUP Rats. Mean (\pm SEM) percent of control levels for hippocampal GFAP mRNA (A) and protein (B) for CON and SUP rats that were treated with saline (solid bars) or KA (hatched bars) and that remained in their home cage (grey) or received additional water maze training (black) following treatment. Both CON and SUP rats showed a significant overall SE-induced increase in GFAP mRNA and protein expression, but this increase was attenuated in KA-treated home cage SUP rats. Repeated water maze training attenuated elevated GFAP protein levels in KA-treated CON rats, and further increased GFAP mRNA levels in KA-treated SUP rats. * significantly different at p < 0.05; ** main effect of seizure revealed by a within diet 2-way ANOVA (seizure × experience), p < 0.05; # KA-treated home cage SUP rats are significantly different from KA-treated home cage CON rats (A, B) and KA-treated water maze CON rats (A).

Long-term survival of dentate cells born shortly after seizures

To examine the long-term (9-10.5 weeks) survival and migration of dentate cells born 5 to 15 days after KA-induced SE, the number of BrdU+ cells in the SGZ-GCL and hilus were visualized and quantified in all rats. Figure 46 shows photomicrographs of BrdU labeling in the hippocampus of representative sections from CON and SUP salineand KA-treated rats (home cage and water maze). BrdU+ cells were expressed throughout the rostral-caudal extent of the dentate gyrus and in both the suprapyramidal and infrapyramidal blades. As can be seen in Figure 46, most BrdU+ cells that were found in the SGZ-GCL had a large and rounded BrdU-immunostained nuclei characteristic of mature granule cells. However, a considerable number of BrdU+ cells were much smaller in size with many cells exhibiting a more elongated oval-shaped nuclei than those found in the GCL, suggesting that many of these hilar BrdU+ cells were putative glial or endothelial cells (Scott et al., 2000; Hellsten et al., 2004). Moreover, a vast majority of BrdU+ cells displayed morphological characteristics of normal nonpyknotic cells (e.g., round or oval nuclei that did not appear highly condensed). Only non-pyknotic cells were counted when quantifying numbers of BrdU+ cells. In KAtreated rats, more BrdU+ cells were present in the hilus in comparison to saline-treated rats (Figure 46A-F).

SE significantly reduced the overall size of hippocampus in both KA-treated CON and SUP rats (as previously stated), but did not appear to significantly alter the volume of the dentate GCL *per se* in either CON rats (saline-treated home cage = 0.67 ± 0.05 mm³; KA-treated home cage = 0.72 ± 0.07 mm³; saline-treated water maze = 0.70 ± 0.02 mm³;



Figure 46. Long-term (9 to 10.5 Weeks) Survival of BrdU-immunopositive Cells Born 6 to 14 Days After Saline or KA Treatment in CON and SUP Rats.

(*A-F*) Photomicrographs of BrdU+ cells in the SGZ-GCL and hilus in saline treated rats (*A*, CON; *B*, SUP) and KA-treated rats who either remained in their home cage (*C*, CON; *D*, SUP) or received additional water maze training (*E*, CON; *F*, SUP) after SE. Most BrdU+ cells that were found in the GCL had large and rounded BrdUimmunostained nuclei characteristic of mature granule cells (large arrows), but many BrdU+ cells in the SGZ-GCL and hilus had morphological features characteristic of glial or endothelial cells (small arrows). KA-treated SUP rats exhibited more BrdU labeling in the SGZ-GCL than KA-treated CON rats. KA-induced SE increased the number of BrdU+ cells in the hilus of all KA-treated rats. Bars indicate 50 μ m. Photomicrographs in first and third columns were taken with a 10x objective and photomicrographs in the second and fourth columns were taken with a 40x objective. GCL, granule cell layer. SGZ, subgranular zone. H, hilus.

KA-treated water maze = $0.78 \pm 0.06 \text{ mm}^3$) or SUP rats (saline-treated home cage = 0.74 ± 0.04 mm³; KA-treated home cage = 0.86 ± 0.07 mm³; saline-treated water maze = 0.74 $\pm 0.01 \text{ mm}^3$; KA-treated water maze = $0.77 \pm 0.05 \text{ mm}^3$). To investigate whether SE altered the overall density of granule cells within the dentate gyrus, a granule cell density measure was generated by estimating the total number of NeuN+ cells in the GCL using the optical fractionator and dividing this estimate by the total GCL volume estimate (number of NeuN+ cells/unit of volume). Importantly, there were no significant differences in granule cell density across treatment groups for CON rats (saline-treated home cage = $231,994 \pm 7,928$ cells/mm³; KA-treated home cage = $248,287 \pm 27,946$ cells/mm³; saline-treated water maze = $253,180 \pm 9,648$ cells/mm³; KA-treated water maze = $239,893 \pm 12,450$ cells/mm³) or SUP rats (saline-treated home cage = $234,181 \pm$ 18,027 cells/mm³; KA-treated home cage = $224,488 \pm 8,015$ cells/mm³; saline-treated water maze = $259,980 \pm 6.223$ cells/mm³; KA-treated water maze = $245,031 \pm 6,290$ cells/mm³). Thus, we can conclude that differences in BrdU+ counts (and DCX+ counts; see next section) across treatment groups within each diet condition are due to differences in the proportion of all granule cells that are BrdU+ and DCX+. Taken together, these data are also consistent with the current observation that the dentate GCL remained relatively intact compared to other cellular subfields in the hippocampus of KA-treated rats (Figure 43), and in line with previous reports showing that dentate granule cells appear to be more resistant than other hippocampal neurons to KA-induced SE (Covolan et al., 2000; Hattiangady et al., 2004).

Interestingly, KA-treated home cage SUP rats had significantly more BrdU+ cells in the SGZ-GCL than KA-treated CON rats in either post-SE experience condition (ps < 10.05; Figure 47A). However, because CON and SUP rats show different rates of SGZ-GCL and hilar cell proliferation shortly after seizures, as demonstrated in Experiment 3 (Wong-Goodrich et al., 2008b), one can not draw conclusions about the effects of prenatal choline supplementation on the long-term survival of granule cells born shortly after seizures by directly comparing numbers of BrdU+ cells (labeled 6 to 14 days after SE) between KA-treated CON and KA-treated SUP rats. Stereological estimates of the number of surviving BrdU+ cells in the SGZ-GCL and hilus were, therefore, analyzed separately for CON and SUP rats via separate 2 (Seizure) \times 2 (Experience) ANOVAs. For CON rats, analyses of the number of BrdU+ cells in the SGZ-GCL revealed a significant main effect of Experience, F(1, 23) = 7.84, p = 0.01, and a significant Seizure \times Experience interaction, F(1, 23) = 4.57, p < 0.05). Post-hoc analyses revealed that KAtreated CON rats that remained in their home cage after seizures had significantly fewer BrdU+ cells (about a 34% reduction) in the SGZ-GCL than all other CON groups (ps < 10.05; Figure 47A). There was no difference between KA-treated CON rats who received post-SE water maze experience and both saline-treated CON rat groups, indicating that post-SE water maze experience rescued the decrease in SGZ-GCL cell survival (Figure 47A). In contrast, KA-treated SUP rats had significantly more BrdU+ cells in the SGZ-GCL than saline-treated SUP rats regardless of post-SE experience, which was confirmed by a main effect of Seizure, F(1, 24) = 8.40, p < 0.01; (Figure 47A). There were no effects of Experience or Diet on the number of BrdU+ cells in the SGZ-GCL of salinetreated rats. Confocal analysis of BrdU+ cells revealed that the majority of BrdU+ cells (~72-90%) found in the SGZ-GCL of all treatment groups were also immunopositive for the mature neuronal marker, NeuN (Figure 48; Table 1). For CON rats, a 2-way ANOVA revealed a main effect of Experience for the percentage of BrdU+/NeuN+ neurons in the dentate gyrus, F(1, 12) = 5.38, p < 0.05, where water maze experience decreased the proportion of surviving BrdU+ cells that differentiated into neurons in both saline- and KA-treated CON rats. Others have also reported decreases in granule cell survival by water maze training, particularly for cells that are in more advanced stages of neuronal development (Ambrogini et al., 2004; Ehninger and Kempermann, 2006; Mohapel et al., 2006). There was no effect of Seizure or Seizure × Experience interaction for CON rats, and no main effects or interaction for SUP rats. Estimated numbers of new neurons in the SGZ-GCL revealed a similar pattern as that of total BrdU+ cells in the SGZ-GCL (Table 1).

The pattern of BrdU labeling in the hilus showed that both CON and SUP KAtreated rats had more BrdU+ cells in the hilus than that of CON and SUP saline-treated rats (Figure 47B). Separate analyses of the total number of BrdU+ cells in the hilus for CON and SUP rats revealed a main effect of Seizure for both CON rats, F(1, 23) = 22.22, p < 0.001, and SUP rats, F(1, 24) = 20.19, p < 0.001, but no significant effect of Experience or Seizure × Experience interaction for either prenatal diet group. KAinduced SE led to a significant increase in the number of BrdU+ cells that were observed in the hilus for all treatment groups, with no significant differences between any KAtreated groups (Figure 47B). In contrast to the pattern of BrdU labeling in the SGZ-GCL,



Figure 47. Quantification of BrdU+ Cells in the Dentate Gyrus 11 Weeks After Saline or KA Treatment in CON and SUP Rats. Mean (\pm SEM) numbers of BrdU+ cells detected in the SGZ-GCL (*A*) and hilus (*B*) of CON and SUP rats that were treated with saline (solid bars) or KA (hatched bars) and that remained in their home cage (grey) or received additional water maze training (black) following treatment. Note that we did not directly compare numbers of BrdU+ cells between KA-treated CON and SUP rats because we have previously found that the amount of SGZ-GCL and hilar cell proliferation observed shortly after seizures is altered by prenatal choline availability (see text). * significantly different at p < 0.05; ** main effect of seizure revealed by a within diet 2-way ANOVA (seizure × experience), p < 0.05.



Figure 48. Confocal Images of BrdU+ Cells Co-labeled with the Mature Neuronal Marker NeuN. Arrow heads indicate BrdU+/NeuN+ cells. Bar indicates 25 µm. Images were taken with a 40x objective. GCL, granule cell layer. H, hilus.

	% NeuN+/BrdU+ in SGZ-GCL	#New Neurons in SGZ-GCL	% NeuN+/BrdU+ in Hilus	#NewNeurons in Hilus
CON				
Saline - Home Cage	85.58 (2.28)	5305.52 (516.24)	**	**
Saline - Water Maze	72.05 (2.86)	5143.62 (554.68)	**	**
KA - Home Cage	85.09 (5.40)	3715.62 (883.20)	53.73 (18.05)	1670.18 (357.33)
KA - Water Maze	78.06 (3.04)	5547.80 (680.28)	46.05 (7.88)	2481.88 (880.87)
SUP				
Saline - Home Cage	85.84 (6.57)	6048.74 (352.82)	**	**
Saline - Water Maze	80.72 (5.75)	6135.98 (339.33)	**	**
KA - Home Cage	90.47 (2.05)	11,311.40 (957.84)	69.27 (4.91)	3571.22 (528.88)
KA - Water Maze	86.58 (2.52)	11, 475.02 (2482.57)	51.55 (14.37)	1597.88 (739.64)

Table 1. Mean (± SEM) Percentage of BrdU+ Cells that Co-Expressed NeuN and Estimated Number of New Neurons as a Function of Prenatal Diet, KA-Induced SE, and Repeated Water Maze Experience.

BrdU+ cells labeled 6 to 14 days following saline or KA treatment were allowed to survive for 9-10.5 weeks. A fraction of BrdU+ cells were analyzed for co-expression of NeuN (n = 4 brains per group). ** Saline-treated rats had very few BrdU+ cells in the hilus and were thus not included in the analysis.

these data reveal that neither prenatal choline supplementation nor water maze training affected the long-term survival of BrdU+ cells born 5-16 days after SE that were observed in the hilus. Relative levels of hilar migration of newborn neurons in KA-treated rats were also assessed and these data are also presented in Table 1. Saline-treated rats had very few BrdU+ cells in the hilus and were not included in the analysis. A 2 (Diet) \times 2 (Experience) ANOVA did not reveal any effect of Diet or Experience on the percentage of BrdU+/NeuN+ neurons in the hilus. More than half of the hilar BrdU+ cells we analyzed in KA-treated CON and KA-treated SUP rats coexpressed NeuN (Table 1). Water maze experience did not significantly alter the estimated number of new neurons in the hilus of KA-treated CON rats, and only a trend toward a reduction in the estimated number of new neurons in the hilus was observed for water maze KA-treated SUP rats (p = 0.07; Table 1). There was, however, much greater within-group variability in percentage of BrdU+/NeuN+ neurons and estimated new neurons in the hilus vs. the SGZ-GCL.

Neurogenesis 11 weeks after KA-induced SE

To examine the neurogenic capacity of the injured hippocampus at 11 weeks following SE, adjacent tissue sections were immunostained to visualize cells immunopositive for the microtubule-associated phosphoprotein, doublecortin (DCX), that is transiently expressed in newly-born neurons that are still in the process of migrating and differentiating (Brown et al., 2003b; Rao and Shetty, 2004). Others have confirmed that DCX expression is a reliable indicator of ongoing neurogenesis in the adult brain (Couillard-Despres et al., 2005). DCX+ cells with processes in various stages of development were evident along the SGZ and GCL in all rats (Figure 49). In all KAtreated rats, DCX+ cells were also present in the hilus, which is consistent with Experiments 3 and 4, and with a previous study demonstrating persistent hilar migration of DCX+ neurons months following KA-induced SE (Hattiangady et al., 2004). In comparison to saline-treated rats, many of the DCX+ neurons in KA-treated rats also appeared displaced and exhibited abnormal morphological features, such as horizontally oriented cell bodies and processes (Figure 49C-F). SUP rats tended to display overall more DCX-labeling than CON rats (Figure 49A-F).

Stereological estimates of the number of DCX+ neurons in the SGZ-GCL were generated for each rat and are shown in Figure 50. Separate 2-way ANOVAs were performed for CON and SUP rats. For CON rats, analyses revealed a significant main effect of Seizure, F(1, 23) = 4.12, p < 0.04, Experience, F(1, 23) = 4.16, p < 0.05, and a significant Seizure × Experience interaction, F(1, 23) = 5.00, p < 0.05. KA-treated CON rats that remained in their home cage after seizures had significantly fewer DCX+ neurons in the SGZ-GCL than saline-treated CON rats (p = 0.01; Figure 50A), which is consistent with a previous report showing decreased DCX+ neurons at 5 months following KA-induced SE (Hattiangady et al., 2004). As can be seen in Figure 50A, 11 weeks after KA-induced seizures, SUP rats showed no difference in the number of DCX+ neurons in the SGZ-GCL compared to saline-treated rats (no significant main effects or Seizure × Experience interaction for SUP rats, Fs < 1). Consistent with previous findings from our laboratory (Glenn et al., 2007), saline-treated SUP rats (~5 months of age at the

time of sacrifice) also exhibited overall higher numbers of DCX+ neurons than salinetreated CON rats, F(1, 26) = 4.80, p < 0.05.

To assess relative levels of hilar migration of DCX+ neurons in KA-treated rats, the number of DCX+ neurons in the hilus was expressed as a percentage of the total number of DCX+ cells (in both the dentate gyrus and hilus), and this measure was subjected to a 2-way ANOVA with Diet and Experience as between-subjects factors. Analyses did not reveal a significant effect of Experience or Diet × Experience interaction, though there was a trend for KA-treated SUP rats to show a lower percentage of DCX+ neurons in the hilus than KA-treated CON rats, F(1, 21) = 3.55, p = 0.07. Planned comparisons revealed that KA-treated SUP rats that received post-SE water maze experience had a lower percentage of DCX+ in the hilus than both KA-treated home cage CON rats and KA-treated water maze CON rats (p < 0.05; Figure 50B). There was no difference between KA-treated CON and SUP rats that remained in their home cage. Similar to the estimates of BrdU+ neurons that migrated to the hilus (Table 1), there was a trend for KA-treated water maze SUP rats to show a lower percentage of hilar migration of DCX+ neurons when compared to KA-treated home cage SUP rats (p =0.07).

Sustained SE-induced increase in hippocampal BDNF protein

Prolonged seizures produce dramatic changes in the hippocampal microenvironment, including altered levels of various neurotrophic/growth factors (Gall, 1993; Rocamora et al., 1994; Marcinkiewicz et al., 1997; Shetty et al., 2003). Protein expression levels of BDNF in the hippocampus were analyzed in each rat and the data are



Figure 49. Hippocampal Neurogenesis 11 Weeks After Saline or KA Treatment in CON and SUP Rats. (*A*-*F*) Photomicrographs of DCX-immunopositive neurons (i.e., newly generated neurons) in the SGZ-GCL and hilus in saline treated rats (*A*, CON; *B*, SUP) and KA-treated rats who either remained in their home cage (*C*, CON; *D*, SUP) or received additional water maze training (*E*, CON; *F*, SUP) after SE. In KA-treated rats of both prenatal diet groups (*C*-*F*), DCX+ neurons were aberrantly located in the hilus and exhibited abnormal morphological features, such as horizontally oriented cell bodies and processes (arrows). SUP rats exhibited more overall DCX labeling than CON rats. Bars indicate 50 μ m. Photomicrographs in first and third columns were taken with a 10x objective and photomicrographs in the second and fourth columns were taken with a 40x objective. GCL, granule cell layer. SGZ, subgranular zone. H, hilus.



Figure 50. Quantification of DCX+ Cells in the Dentate Gyrus 11 Weeks After Saline or KA Treatment in CON and SUP Rats. Mean (±SEM) numbers of DCX+ cells detected in the SGZ-GCL (A) and mean percentage of DCX+ found in the hilus (B) of CON and SUP rats that were treated with saline (solid bars) or KA (hatched bars) and that remained in their home cage (grey) or received additional water maze training (black) following treatment. (A) CON rats showed a significant decrease in the number of DCX+ cells in the SGZ-GCL, which was rescued by repeated water maze training. KA-treated SUP rats had preserved levels of DCX+ cells in the SGZ-GCL, regardless of post-SE experience. Saline-treated SUP rats also had a higher number of DCX+ neurons overall than saline-treated CON rats. (B) Both KA-treated CON and SUP rats generated a proportion of DCX+ cells that migrated to the hilus. KA-treated water maze SUP rats had a significantly lower percentage of DCX+ neurons in the hilus than KA-treated home cage and water maze rats (#, p < 0.05). * significantly different at p < 0.05; ** main effect of seizure revealed by a within diet 2-way ANOVA (seizure \times experience), p < 0.05; ## main effect of prenatal diet revealed by a within diet 2-way ANOVA (diet \times experience), p < 0.05.

presented in Figure 51. Analyses revealed a main effect of Seizure for both CON rats, F(1, 23) = 15.30, p < 0.01, and SUP rats, F(1, 23) = 25.79, p < 0.001, where KA-treated rats showed elevated levels of BDNF protein compared to saline-treated rats. There was no effect of Experience or Seizure × Experience interaction for either CON or SUP rats, but there was a trend for SE to induce a greater increase in BDNF in home cage SUP rats than home cage CON rats (p = 0.09; Figure 51). Consistent with Experiments 1 and 3, and with previous findings from our lab (Glenn et al., 2007), analysis of saline-treated rats revealed that SUP rats exhibited overall higher levels of BDNF protein (about 30% more) than CON rats, F(1,26) = 5.06, p < 0.05.

Discussion

Experiment 5 revealed that prenatal choline supplemented rats that had undergone kainic acid-induced status epilepticus in young adulthood had attenuated spatial learning deficits and a complete recovery of spatial memory retention by 10 weeks after SE. This prophylactic dietary manipulation also prevented or mitigated long-term alterations in GABAergic function, reactive astrogliosis, and hippocampal neurogenesis that were present nearly 3 months after SE and that are hypothesized to contribute to the development of chronic epilepsy and ensuing learning and memory impairments. These data add to the growing literature that point to the important role of perinatal choline intake in neural protection (Guo-Ross et al., 2002; Holmes et al., 2002; Guo-Ross et al., 2003; Thomas et al., 2004; Nag and Berger-Sweeney, 2007; Thomas et al., 2007; Glenn et al., 2008b; Meck et al., 2008; Thomas et al., 2009). The current experiment also reports, for the first time, that repeatedly engaging in a hippocampally-mediated water



Figure 51. Hippocampal BDNF Expression 11 Weeks After Saline or KA Treatment in CON and SUP Rats. Mean (\pm SEM) percent of control levels for hippocampal BDNF protein for CON and SUP rats that were treated with saline (solid bars) or KA (hatched bars) and that remained in their home cage (grey) or received additional water maze training (black) following treatment. Both CON and SUP rats showed a significant overall SE-induced increase in BDNF protein. Saline-treated SUP rats also had a higher levels of BDNF protein overall than saline-treated CON rats. * significantly different at p < 0.05; ** main effect of seizure revealed by a within diet 2-way ANOVA (seizure × experience), p < 0.05; # main effect of prenatal diet revealed by a within diet 2-way ANOVA (diet × experience), p < 0.05.

maze task following SE is beneficial for long-term hippocampal recovery. For example, repeated post-SE water maze training rescued SE-induced declines in GAD mRNA and dentate neurogenesis, and attenuated elevated GFAP protein levels. Consistent with Experiments 1 and 3 and with previous reports (Glenn et al., 2007), prenatal choline supplemented rats had a larger baseline pool of hippocampal BDNF and increased hippocampal neurogenesis. Enhanced adult hippocampal plasticity and trophic support may have provided a more permissive hippocampal microenvironment that enabled the prenatal choline supplemented adult hippocampus to better withstand the long-term consequences of SE, and possibly respond more effectively to the rehabilitative effects of post-SE water maze training. The following is a discussion of the long-term rehabilitative (water maze training) and prophylactic (prenatal choline supplementation) treatments altered the long-term effects of SE, and the potential implications of these altered outcomes for cognitive and neural recovery.

Long-term effects of KA-induced SE in the adult hippocampus

Although widespread neurodegeneration and altered plasticity of the hippocampus is observed months following excitotoxic injury-induced SE, GABAergic neurons in the hippocampus are especially vulnerable (similar to short-term effects of prolonged seizures; see Chapter 3). SE can lead to a loss of some types of inhibitory neurons, changes in GAD content, and altered GABA receptor expression, all of which are thought contribute to epileptogenesis (Sloviter, 1987; Davenport et al., 1990; Kash et al., 1997; Esclapez and Houser, 1999; Morimoto et al., 2004; Sperk et al., 2004; Brooks-Kayal et al., 2009). The finding that hippocampal GAD65/67 mRNA and protein levels were decreased at nearly 3 months after KA-induced SE is consistent with previous reports that have shown a significant reduction in GAD65- and GAD67-containing neurons (cell bodies and terminals) in all cellular subfields of the hippocampus at 1 to 6 months after excitotoxic injury/seizures (Houser and Esclapez, 1996; Esclapez and Houser, 1999; Shetty and Turner, 2001; Dinocourt et al., 2003; Shetty et al., 2009). Using in situ hybridization and immunohistochemical techniques, it has also been demonstrated that the remaining GAD-containing neurons in the hippocampus, including interneurons and dentate granule cells, show continued upregulation of GAD65 and 67 mRNA and immunoreactivity months after injury (Feldblum et al., 1990; Houser and Esclapez, 1996; Sloviter et al., 1996; Esclapez and Houser, 1999; Knopp et al., 2008). The present experiment quantified total hippocampal GAD65 and 67 mRNA and protein content, but the current techniques did not allow us to localize the distribution of GAD mRNA and protein. Thus, it is possible that the decrease in total GAD content that was observed in KA-treated CON rats masked a selective upregulation of GAD in the remaining GABAergic neurons that were preserved months after SE.

Increased hippocampal levels of GFAP may also play a role in hyperexcitability and structural reorganization of the hippocampus after SE that may underlie development of epilepsy. In addition to altered membrane channel, receptor, and transporter properties of astrocytes following SE that might add to neuronal hyperexcitability (Jabs et al., 2008), GFAP-expressing reactive astrocytes express neurite growth-promoting genes thought to support aberrant mossy fiber excitatory innervation of the dentate gyrus (Represa et al., 1993; Niquet et al., 1994a; Niquet et al., 1994b; Khrestchatisky et al., 1995; Stringer, 1996), which occurs prior to the onset of spontaneous motor seizures and may generate recurrent excitation underlying the development of seizures (Koyama and Ikegaya, 2004; Sharma et al., 2008). In the present experiment, elevated hippocampal GFAP mRNA and protein persisted for almost 3 months after KA-induced SE, which is consistent with elevated hippocampal GFAP immunoreactivity at 1 to 3 months after experimental SE (Aronica et al., 2000; Bendotti et al., 2000; Borges et al., 2006) and elevated GFAP in association with mossy fiber sprouting in the human epileptic hippocampus (Proper et al., 2000).

It is well established that seizures stimulate a vast proliferation of adult neural progenitors in the dentate gyrus within 1-3 days of SE (Bengzon et al., 1997; Parent et al., 1997; Gray and Sundstrom, 1998; Parent et al., 1999; Jessberger et al., 2005; Steiner et al., 2008), many of which become mature neurons and survive for weeks to months after SE (Parent et al., 1997; Madsen et al., 2000; Bonde et al., 2006); a profound number undergo apoptotic cell death by at least 4 weeks after cell division (Ekdahl et al., 2001; Ekdahl et al., 2002). Beyond 4 weeks, little is known about the survival rate of seizure-induced dentate granule cells born just 1-2 weeks after experimental SE. The results of the current experiment report a decrease in the ~2.5 month survival of BrdU+ cells in the SGZ-GCL that were labeled 6 to 14 days after SE, compared to the survival of SGZ-GCL BrdU+ cells from saline-treated rats, with a majority of these cells expressing a mature neuronal phenotype. In contrast, SE increased BrdU labeling in the dentate hilus, which suggests that the survival rates of newborn dentate cells born shortly after SE that migrate

to the hilus versus GCL may operate via different mechanisms. Indeed, prior work has demonstrated that a loss in reelin following SE (via death of reelin-producing hilar interneurons) leads to aberrant hilar migration of dentate granule cell precursors (Gong et al., 2007). Moreover, blocking caspase-mediated cell death 7 days after SE selectively increases the survival of newborn dentate cells born shortly after seizures that migrate to the SGZ-GCL, but not to the hilus (Ekdahl et al., 2002), perhaps due to a savings in hilar reelin levels (Gong et al., 2007). The current experiment's findings are also consistent with a previous study showing that SE reduced the number of BrdU+/NeuN+ granule cells that were born 1 week after SE and observed in the SGZ-GCL 4 weeks later, despite an increase in the number of BrdU+ cells present in the hilus (Yang et al., 2008). However, others have also reported an increase in the survival of SGZ-GCL BrdU+/NeuN+ neurons born shortly after SE, using various other models (Ekdahl et al., 2003; Mohapel et al., 2004; Bonde et al., 2006; Jessberger et al., 2007a). The current experiment's findings suggest that there may be continued cell death of seizure-induced dentate granule cells in the GCL-SGZ beyond 4 weeks in this injury model. Notably, more severe, fully convulsive SE (as produced in the present experiment) has been shown to reduce the long-term survival of dentate granule cells born 1 week after SE in comparison to granule cells born in response to more mild seizures (Mohapel et al., 2004; Yang et al., 2008). Thus, differences in models of SE may account for the disparate findings reported in the present experiment.

Similar to the pattern of BrdU labeling found in the current experiment, it was also found that at nearly 3 months after SE, there was a decrease in the number of DCX+

neurons in the SGZ-GCL with a concomitant appearance of DCX+ neurons in the hilus. Long-term declines in dentate neurogenesis in models of temporal lobe epilepsy have been previously reported (Hattiangady et al., 2004; Kralic et al., 2005; Heinrich et al., 2006; Hattiangady and Shetty, 2010), though others have also reported no decline (Jessberger et al., 2007b). The current findings are also supported by similar declines found in hippocampus tissue ressected from temporal lobe epilepsy patients (Mathern et al., 2002; Parent et al., 2006; Fahrner et al., 2007). Despite the reduction in dentate neurogenesis at nearly 3 months post-SE, a sustained increase in hippocampal BDNF protein levels was observed, which others have also reported weeks to months after experimental SE (Vezzani et al., 1999; Revuelta et al., 2001; Scharfman et al., 2002b; Sartori et al., 2009) as well as in the human epileptic hippocampus (Mathern et al., 1997; Takahashi et al., 1999; Murray et al., 2000). Future studies are needed to clarify whether or not elevated hippocampal BDNF following SE and in the epileptic brain is neuroprotective or detrimental to functional recovery, as accumulating evidence has provided support for both theories (Binder et al., 2001; Koyama and Ikegaya, 2005; Hattiangady and Shetty, 2008b).

Repeated water maze testing and prenatal choline supplementation aid recovery from SE

The current experiment reports that despite no significant differences in seizure severity and progression during SE induction across KA-treated groups and similar putative levels of hippocampal damage, repeated water maze testing and prenatal choline supplementation differentially promoted the long-term hippocampal recovery from KAinduced SE. While many others have assessed cognitive function during a specific time point after experimental SE and have found that cognition is compromised weeks to months following SE (Stafstrom et al., 1993; Liu et al., 1994; Sarkisian et al., 1997; Hort et al., 1999; Mikati et al., 2001; Kemppainen and Pitkanen, 2004; McKay and Persinger, 2004; Detour et al., 2005; Lin et al., 2009; Sartori et al., 2009), the present experiment is the first to examine whether repeatedly engaging the hippocampus in a cognitive task would promote the long-term recovery from SE. Spatial learning in the water maze offers an experience that engages the hippocampus (Jenkins et al., 2004; Kee et al., 2007) and provides animals with a learning, enrichment, and exercise component—all of which have been shown to enhance hippocampal plasticity and cognitive function in the intact and/or injured brain (Gould et al., 1999a; van Praag et al., 2000; Gobbo and O'Mara, 2004; Gobbo and O'Mara, 2005; Cotman et al., 2007; Sartori et al., 2009), suggesting that this experience may be a good candidate for facilitating some aspects of recovery from SE. It is important to note that after SE, our water maze trained CON rats still showed poor spatial learning and no evidence of memory retention recovery over 3 weeks. However, water maze-trained rats in the current experiment only received a total of 15 days of intermittent testing in a span of 11 weeks after SE. Perhaps more prolonged and uninterrupted exposure to cognitive testing after SE would have facilitated a better cognitive recovery. It is also possible that spatial learning and memory retention were rehabilitated to some degree with repeated water maze testing in KA-treated CON rats, as post-excitotoxic injury enrichment and exercise has been shown to only attenuate, not completely abolish, cognitive deficits (Gobbo and O'Mara, 2004; Gobbo and O'Mara, 2005; Sartori et al., 2009). As such, the cognitive deficits observed at 10 weeks post-SE

might have been further exacerbated in KA-treated CON rats that remained in their home cage (though home cage rats were not tested in order to preserve a true post-SE home cage condition for comparison of brain measures).

Despite poor cognitive performance, the experience of water maze training in KA-treated CON rats prevented declines in hippocampal GAD mRNA, attenuated the SE-induced increase in GFAP protein, and preserved levels of dentate neurogenesis. A lack of obvious behavioral benefit from repeated water maze experience despite restorative effects on the hippocampus may also suggest that these specific neural outcomes may not be sufficient for restoring spatial learning and memory function following SE. For example, there is evidence that hippocampal neurogenesis appears to be important for some, but not all types of spatial learning and memory function (Shors et al., 2002; Madsen et al., 2003; Snyder et al., 2005; Saxe et al., 2006; Deng et al., 2009), and that neurogenesis in the epileptic brain may actually be detrimental (Scharfman and Gray, 2007). Future studies using SE models are needed to determine whether the recovery of newborn granule cells in an epileptic brain directly contribute to recovery of cognitive function. Alternately, other extrahippocampal areas that have been implicated in aspects of learning and memory are also severely damaged following KA-induced SE, such as the pyriform cortex, entorhinal cortex, septum, and amygdala (Schwob et al., 1980; Heggli et al., 1981; Sperk et al., 1983). These areas were not examined in the current experiment, but the possibility that SE-induced changes in these extrahippocampal areas may contribute to cognitive outcomes can not be excluded.

The present experiment is also the first to investigate whether the protective effects of prenatal choline supplementation against the sequelae of seizures are longlasting. It was found that prenatal choline supplementation alone mitigated the long-term consequences of SE to an even greater extent than that of water maze experience: SUP rats that remained in their home cage after SE were protected against losses in GAD mRNA and GAD65 protein, showed attenuated seizure-induced elevations in both GFAP mRNA and protein, and did not show deficits in dentate neurogenesis. That preserved levels of GAD and attenuated GFAP expression were found in KA-treated SUP rats at nearly 3 months after SE extends the initial findings from Experiment 3 that revealed a similar pattern of protection in KA-treated SUP rats at just 16 days after SE (Wong-Goodrich et al., 2008b), indicating that prenatal choline supplementation's protective effects on these measures are remarkably long-lasting. In the present experiment, it was also discovered that KA-treated SUP rats showed an increase in the number of BrdU+ granule cells in the SGZ-GCL compared to saline-treated SUP rats, which was surprising given that there was no such increase in water maze KA-treated CON rats and that Experiment 3 demonstrated an attenuation of BrdU+ cells in the SGZ-GCL at just 16 days after SE in KA-treated SUP rats (Wong-Goodrich et al., 2008b). It is difficult to directly compare numbers of BrdU+ cells between these two experiments, given the differences in BrdU regimens. However, taken together, Experiment 3's findings and current findings suggest that 1) many of these newly generated cells labeled 6 to 14 days after SE likely undergo cell death 2.5 months later, and 2) in addition to attenuated dentate cell proliferation shortly after seizures (Experiment 3, Wong-Goodrich et al.,

2008b), prenatal choline supplementation may also lessen the magnitude of cell death occurring weeks to months after KA-induced SE. Prenatal choline supplementation has been found to reduce relative levels of apoptotic cell death in the fetal hippocampus (Albright et al., 1999; Craciunescu et al., 2003), and we recent work from our lab and our colleagues has discovered that levels of apoptotic cell death in the adult hippocampus are reduced in aged prenatal choline supplemented rats (unpublished observations, M.J., Glenn, M.D. Niculescu, C.L. Williams, and S.H. Zeisel). It is therefore possible that prenatal choline supplementation also reduced the amount of dentate granule cell death following seizures, although this remains to be determined.

Protection (or recovery) from post-SE disruptions in hippocampal GAD, GFAP, and granule cell survival may have implications for ameliorating the epileptogenic outcome of SE. Preventing losses in GAD (via protecting against the death of GABAergic neurons and/or increasing expression of GAD in remaining GABAergic neurons) may restore GABAergic function in the injured hippocampus (Houser and Esclapez, 1996; Esclapez and Houser, 1999; Shetty and Turner, 2001; Dinocourt et al., 2003; Shetty et al., 2009). Attenuated hippocampal GFAP expression following SE may also help reduce hyperexcitability. Indeed, the magnitude of reactive gliosis may be a reliable predictor of epileptic seizure severity after experimental SE (Somera-Molina et al., 2007) and in human epilepsy patients (Guerreiro et al., 2003). Interestingly, the protective effects of repeated water maze experience and prenatal choline supplementation on the survival of granule cells born shortly after SE appear to be specific to dentate granule cells that migrate to the GCL. This finding is compelling

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because dentate granule cells born 1 week after SE that migrate properly to the GCL show reduced excitability compared to mature granule cells, and may therefore serve a restorative function in counteracting the hyperexcitability that underlies epilepsy (Jakubs et al., 2006). Thus, enhanced granule cell survival in the GCL in particular may be especially beneficial to recovery from SE. The number of spontaneous motor seizures that are observed following SE using the model employed in the current experiment are apparent as early as one month after SE induction, and become increasingly more apparent during the third through sixth month after SE (Hellier et al., 1998; Hattiangady et al., 2004; Williams et al., 2009). Spontaneous motor seizure activity was observed in very few of our KA-treated rats in the present experiment that were euthanized shortly before 3 months post-SE, but extensive observations of spontaneous seizure activity were not performed, nor was EEG activity monitored. It is possible that the number of rats having spontaneous seizures was underestimated, or that epileptic seizures would have manifested at a later time point, which may also make interpreting some of these findings difficult. For example, spontaneous epileptic seizures has been shown to enhance BDNF immunoreactivity in the hippocampus (Vezzani et al., 1999), and a higher frequency of spontaneous seizures has been linked with a more pronounced decline in neurogenesis months after SE (Hattiangady et al., 2004). Whether the protective effects of water maze testing and prenatal choline supplementation on brain and behavior pertain to the development of epileptic seizures, however, remains to be determined.

The present experiment also showed that both repeated water maze experience and prenatal choline supplementation also rescued the decline in the number of newborn neurons present at nearly 3 months after KA-induced SE. While it is not clear whether aberrantly high levels of dentate neurogenesis directly contribute to cognitive deficits observed shortly after SE, it has been suggested that the subsequent downregulation of dentate neurogenesis weeks to months after the initial injury may contribute to cognitive deficits associated with epilepsy (Mikati et al., 2001; Elger et al., 2004; Hattiangady et al., 2004; Dhanushkodi and Shetty, 2008; Hattiangady and Shetty, 2008b). Remarkably, prenatal choline supplementation attenuated the spatial learning deficits elicited by SE and completely restored spatial memory retention ability to that of saline-treated rats by 10 weeks after SE. These findings suggest that the protection of spatial learning and memory function conferred by prenatal choline supplementation extends much further beyond 1-2 weeks after SE (Yang et al., 2000; Holmes et al., 2002). Recovery of neurogenesis in SUP rats months after seizures also appeared to be independent of post-SE water maze training, as home cage KA-treated SUP rats also showed a preservation of DCX+ neurons. However, it has been suggested that aberrant migration of newborn neurons to the hilus may contribute to epileptogenesis and cognitive deficits after SE (Parent et al., 1997; Scharfman et al., 2000; Scharfman and Gray, 2007; Scharfman and Hen, 2007). Although neither repeated water maze testing nor prenatal choline supplementation significantly altered the number of ectopic hilar granule cells born shortly after SE, there was a trend for SUP rats who received additional water maze training after SE to show reduced BrdU+ neurons in the hilus compared to SUP rats that remained in their home cage after SE. A similar trend was observed on the percentage of DCX+ neurons that migrated to the hilus, but it is still not definitive whether the effects

of prenatal choline and water maze experience are additive. When comparing KA-treated CON versus SUP rats that received post-SE water maze experience, hilar migration of newborn DCX+ neurons at 3 months post-SE was significantly reduced in SUP rats. Given that these two groups both showed a similar recovery of the number of DCX+ neurons at 3 months after SE, perhaps it is the recovery in migratory behavior of newborn dentate granule that may have contributed to the improved spatial learning and memory retention ability observed in KA-treated SUP rats. Future studies will further explore whether prenatal choline supplementation can enable the injured adult hippocampus to more effectively respond to the rehabilitative effects of cognitive enrichment.

Protective effects of prenatal choline supplementation

Similar to Experiment 3, it is hypothesized that enhanced adult hippocampal plasticity and neurotrophic milieu prior to injury may underlie prenatal choline supplementation's cognitive enhancing effects and neuroprotection of the adult hippocampus. That KA-treated CON rats in Experiment 5 did not show recovery of spatial learning and memory may further support the notion that factors that affect the hippocampal microenvironment prior to injury may be extremely important predictors of cognitive recovery. The present experiment found that prenatal choline supplementation increased the number of dentate DCX+ neurons and hippocampal BDNF protein in saline-treated adult rats, which is in concert with Experiments 1 and 3, and a previous study from our laboratory (Glenn et al., 2008b). BDNF may be especially relevant for the long-term neural recovery following SE, including protection of GABAergic interneurons (Prince et al., 2009), reducing hippocampal neuronal damage and spontaneous seizures,

and rescuing dentate neurogenesis (Paradiso et al., 2009). Lifelong enhanced hippocampal plasticity and trophic support may render the hippocampus better able to withstand or recover from a neural insult. Interventions that have been shown to enhance hippocampal plasticity, such as cognitive enrichment and early choline availability, may thus provide helpful insights into the development of treatments for the long-term consequences of SE and epilepsy.

GENERAL DISCUSSION

The goal of this dissertation research was to investigate how the availability of a single vital nutrient, choline, *in utero* alters properties of neurogenesis in the adult rat brain (in the intact brain and in response to seizures), and attempt to relate changes in adult hippocampal neurogenesis to changes in learning and memory function. It was hypothesized that increases in neurogenesis and trophic support in the adult hippocampus is one (of likely many) candidate mechanisms for the memory-enhancing and neuroprotective actions of prenatal choline supplementation.

Summary of Findings

The current dissertation work revealed that altered prenatal choline availability modulates adult neurogenesis in the both the intact and injured brain, suggesting one potential mechanism for prenatal choline's effects on adult hippocampal memory function throughout life and in the context of neural insult. Experiment 1 revealed that altered prenatal choline availability led to changes in properties of basal neurogenesis in the adult hippocampus of female rats: prenatal choline supplementation increased the number of newly born cells and their long-term survival in the adult dentate gyrus, the number of proliferating neural stem/progenitor cells in the SGZ, and the proportion of Type-1 radial glia-like stem cells in the SGZ—all of which likely contributed to a dentate gyrus with more new neurons for use in its circuitry. In contrast, prenatal choline deficiency did not alter basal levels of cell division in the dentate gyrus, but did slightly reduce rates of neuronal differentiation, which translated to a net decrease in the population of new neurons in the adult dentate gyrus. Prenatal choline supplementation's enhancement of adult hippocampal neurogenesis was associated with improvements in spatial working memory retention and increased hippocampal neurotrophic factor (e.g., BDNF and NGF) expression. These findings suggest that prenatal choline supplementation may be acting via several mechanisms to alter the number of newborn neurons in the adult hippocampus: changes in the population of stem/progenitor cells, increased cell division, and maintained survival of a larger population of migrating and maturating cells. These alterations in the plasticity of the adult dentate circuitry may contribute to changes in memory precision and capacity, leading to altered memory function throughout life.

Similar to the hippocampus, prenatal choline supplementation increased the number of newborn neurons observed in the adult olfactory bulb, while prenatal choline deficiency had no effect (Experiment 2). This increase in the number of new neurons in the olfactory bulb occurred without altering cell proliferation in the SVZ or neurotrophic factor expression in the olfactory bulb. These findings indicate that prenatal choline's effects on adult neurogenesis are not region-specific and provide support for an important role of the basal forebrain cholinergic system in prenatal choline's enduring effects on adult neurogenesis.

In addition to altering neurogenic properties of the intact adult brain, altered prenatal choline availability also modulated the hippocampal response to seizures where supplementation attenuated (Experiment 3) while deficiency had no effect (Experiment 4) on the injury-induced proliferative response of the dentate gyrus shortly after excitotoxic injury. Prenatal choline supplementation also attenuated other markers of neuropathology
shortly after seizures (Experiment 3) and promoted the long-term hippocampal recovery from seizures months after excitotoxic injury, including rescuing declines in adult hippocampal neurogenesis and in spatial memory (Experiment 5). These findings demonstrate a robust neuroprotective effect of prenatal choline supplementation that may be driven by enhanced hippocampal plasticity and trophic support prior to injury, as demonstrated here (Experiments 1, 3, and 5) and elsewhere (Pyapali et al., 1998; Jones et al., 1999; Sandstrom et al., 2002; Mellott et al., 2004; Glenn et al., 2007; Glenn et al., 2008b; Meck et al., 2008; Wong-Goodrich et al., 2008a).

Developmental Mechanisms for the Lasting Effects of Prenatal Choline Availability on Adult Brain and Behavior

The neurochemical and molecular mechanisms by which altered choline availability *in utero* leads to lasting changes in memory and hippocampal plasticity throughout adulthood (in the intact and injured brain) are currently not well understood in part because choline serves several biological functions. As stated in the General Introduction, choline is the precursor for the neurotransmitter, acetylcholine (ACh), the structural phospholipids in biological membranes, phosphatidylcholine and sphingomyelin, and two cell signaling lipids, sphingosylphosphocholine and plateletactivating factor (Blusztajn, 1998; Zeisel, 2006). Choline also serves as a methyl donor after its oxidization to betaine (Cohen and Wurtman, 1975; Blusztajn and Wurtman, 1983). It is possible that choline availability *in utero* is working via one or more of these pathways during brain development, resulting in changes in the brain's organization.

DNA and histone methylation

One developmental mechanism for the enduring actions of prenatal choline availability on brain function may be through an epigenetic process. Thus, choline as the precursor to the methyl donor betaine may be a key contributing factor, as nutritional methyl status influences the availability of methyl groups necessary for the methylation of CpG sites in DNA or methylation of histories (Blusztajn and Wurtman, 1983; Blusztajn, 1998; Zeisel, 2009), and thus regulation of gene expression, including specific genes relevant for hippocampal plasticity. Indeed, prenatal choline availability during ED 12-17 determines ED 18 brain betaine concentrations (Garner et al., 1995), influences global, gene-specific, and CpG site-specific DNA methylation as well as global and genespecific histone methylation in the fetal brain (Niculescu et al., 2004; Niculescu et al., 2006; Kovacheva et al., 2007; Davison et al., 2009; Mehedint et al., 2010). Recent evidence has now shown that altered prenatal choline availability modulates global and gene-specific histone methylation in the fetal hippocampus in vivo and in ED 14 neural precursor cells *in vitro*, which results in changes in expression of genes that regulate neurogenesis and apoptosis in the fetal hippocampus (Mehedint et al., 2010).

Prenatal choline-related epigenetic modifications of the fetal brain may developmentally program the expression of relevant genes that may lead to lasting alterations in adult brain plasticity and cognitive function. This possibility is appealing because altered gene expression in stem/progenitor cells is a reasonable mechanism by which a permanent change in mitosis might be developmentally programmed. For example, when pregnant rodents are fed a choline deficient diet, methylation of the CDKN3 gene promoter is decreased in fetal brain, which results in over expression of this gene, leading to decreased cell proliferation (Niculescu et al., 2004). Thus, a developmental change in gene promoter methylation by prenatal choline supplementation or deficiency could lead to the long-term alterations in adult hippocampal cell proliferation/neurogenesis reported in the current dissertation research and elsewhere (Glenn et al., 2007; Wong-Goodrich et al., 2008a), as well as in other types of hippocampal plasticity. Indeed, altered prenatal choline availability causes multiple modifications in the postnatal developmental expression patterns of multiple genes both relevant for hippocampal plasticity and known to influence learning and memory, which provide molecular correlates for the cognitive changes evoked by altered availability of choline *in utero* (Mellott et al., 2007).

Metabolic imprinting of cholinergic function

While it is unknown whether prenatal choline availability alters the brain and behavioral changes reported here via alterations in DNA and/or histone methylation, or through some alternate mechanism that influences the development of cholinergic neurons, there is considerable evidence that choline availability *in utero* leads to multiple changes in its own metabolism (i.e., exerts a form of metabolic imprinting) later in life (Blusztajn et al., 1998; Cermak et al., 1998; Cermak et al., 1999). As stated previously, prenatal choline availability produces long-term adaptations in the synthesis, storage and release of ACh, and reuptake and recycling of choline in the adult hippocampus (Blusztajn et al., 1998; Cermak et al., 1998; Cermak et al., 1999) as well as alterations in the size and shape of basal forebrain cholinergic neurons (Williams et al., 1998; McKeon-O'Malley et al., 2003). For example, prenatally choline supplemented rats compared to control-fed rats have relative slow ACh turnover (intrasynaptic ACh concentrations and dwell times are increased) and depolarization-evoked ACh release is very high in the adult hippocampus (Blusztajn et al., 1998; Cermak et al., 1998; Cermak et al., 1999; Meck et al., 2008), which suggest that cholinergic neurotransmission is well maintained and the pool of choline used for the synthesis of ACh in these animals may include that stored in membrane phosphatidylcholine (Wecker, 1986; Ulus et al., 1989). Cell bodies of basal forebrain cholinergic neurons that project to the hippocampus are also larger in prenatally choline supplemented adult rats (Williams et al., 1998).

It is likely that the outcome of metabolic imprinting of cholinergic function in prenatally choline supplemented rats contributes to the effects of altered prenatal choline availability on adult neurogenesis, neuroprotection, and behavior reported in the current dissertation research for several potential reasons. First, the cholinergic system has a central role in memory function (Bartus et al., 1982; Fibiger, 1991; Hasselmo and Giocomo, 2006) and prenatal choline supplementation leads to lifelong improvements in memory precision and capacity, as previously reported (Meck et al., 1988; Meck et al., 1989; Williams et al., 1998; Meck and Williams, 1999; Meck and Williams, 2003; Meck et al., 2008; Wong-Goodrich et al., 2008a) and demonstrated here (Experiment 1). Second, the cholinergic system appears to regulate adult neurogenesis in both the hippocampus and olfactory bulb (Cooper-Kuhn et al., 2004; Mohapel et al., 2005; Kaneko et al., 2006; Kotani et al., 2006; Itou et al., in press). Prenatal choline supplementation, which enhances cholinergic transmission, modulates adult neurogenesis in the same capacity as that of pharmacological manipulations (i.e., agonists) to the adult cholinergic system: increasing adult hippocampal neurogenesis (Experiment 1) and having no effect on SVZ cell proliferation while increasing the number of newborn neurons observed in the olfactory bulb (Experiment 2). Third, the larger pool of neurotrophic/growth factors in the prenatally choline supplemented hippocampus (Experiments 1, 3, and 5), which are hypothesized to support increased basal levels of adult neurogenesis and provide neuroprotection against neural assault (i.e., seizures), may be due to enhanced cholinergic neurotransmission (Blusztajn et al., 1998; Cermak et al., 1998; Cermak et al., 1999; Meck et al., 2008) and increased responsiveness to cholinergic stimulation (Montoya et al., 2000) in the adult hippocampus, as these factors are also known to upregulate hippocampal BDNF and NGF expression (Lindefors et al., 1992; Knipper et al., 1994). Fourth, prenatal choline supplementation also enhances the phosphorylation of CREB in the adult hippocampus (Mellott et al., 2004) and CREB signaling, which regulates both adult hippocampal neurogenesis (Nakagawa et al., 2002; Fujioka et al., 2004) and BDNF transcription (Tao et al., 1998), can be directly modified by manipulations to the cholinergic system. Cholinergic agonists enhance while cholinergic antagonists suppress the phosphorylation of CREB in the adult dentate gyrus (Kotani et al., 2006; Kotani et al., 2008). Thus, enhanced cholinergic transmission conferred by prenatal choline supplementation may also work through CREB signaling pathways to enhance adult neurogenesis and neurotrophic factor expression in the hippocampus. For all these reasons, it seems likely that changes in cholinergic function programmed during early development by prenatal choline availability contribute to the

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diet-induced changes in adult brain and behavior reported in the current dissertation research. However, additional studies are needed to directly test these hypotheses.

In contrast, prenatally choline deficient rats compared to control-fed rats have accelerated ACh turnover (i.e., there is more rapid synthesis, degradation, and choline reutilization), reduced ACh content, and relative inability to sustain depolarizationevoked ACh release in the adult hippocampus (Blusztajn et al., 1998; Cermak et al., 1998; Cermak et al., 1999; Meck et al., 2008) as well as smaller basal forebrain cholinergic neurons that project to the hippocampus (McKeon-O'Malley et al., 2003). Efficient recycling of ACh in the synapse may be one mechanism underlying the comparable (and sometimes superior) cognitive performance of prenatally cholinedeficient rats to that of control-fed rats when the cognitive load is minimal, as demonstrated in Experiment 1 and elsewhere (Meck and Williams, 1999; Meck and Williams, 2003; Wong-Goodrich et al., 2008a), and may help sustain comparable basal levels of cell division/neurogenesis in the adult brain to that of control-fed rats (Experiments 1 and 2; Glenn et al., 2007). However, efficient ACh recycling and lower ACh content is not likely to be able to provide sufficient ACh release when cognitive load is very high, which may explain why prenatally choline-deficient rats show exacerbated cognitive deficits compared to control-fed rats when task demands are increased (Meck and Williams, 1997c; Meck and Williams, 1999; Meck and Williams, 2003). Moreover, cholinergic input appears to be important for experience-induced adult neurogenesis in adult rodents, as lesions of basal forebrain cholinergic neurons abrogates both exercise- and enrichment-induced hippocampal neurogenesis (Frechette et al., 2009;

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Itou et al., in press). Thus, reduced ACh release in prenatally choline deficient rats may also not be sufficient to support reactive neurogenesis in the intact adult hippocampus in response to enriching experiences (Glenn et al., 2007), or perhaps in response to other kinds of experience-derived neurogenic stimuli (e.g., physical activity, learning, enriched odor exposure, etc.)

It is unknown whether cholinergic input is necessary for seizure-induced neurogenesis in the hippocampus, but given the BrdU and DCX results of Experiment 4 and the marked increase in hippocampal neurotrophic/growth factor expression and astrogliosis that accompany status epilepticus in prenatally choline deficient rats, it is predicted that these additional molecular signals likely override a lack of sufficient ACh release conferred by prenatal choline deficiency to yield similar levels of seizure-induced neurogenesis when compared to control-fed rats. This override in prenatally choline deficient animals may, however, occur with additional consequences. Given that newborn neurons in the adult hippocampus express various ACh receptor subtypes (Mohapel et al., 2005; Kaneko et al., 2006; Itou et al., in press), it is reasonable to predict that robust increases in adult neurogenesis due to seizures may increase the demand for cholinergic input to the hippocampus. Notably, prenatally choline-deficient rats do show reduced hippocampal ChAT (enzyme involved in ACh synthesis) activity shortly after SE compared to KA-treated control rats (Holmes et al., 2002), suggesting decreased availability of ACh for cholinergic transmission shortly after seizures. Taken together with the findings from Experiment 4, one hypothesis is that perhaps because prenatally choline deficient rats already have lower hippocampal ACh content prior to seizures

(Blusztajn et al., 1998; Cermak et al., 1998; Cermak et al., 1999; Meck et al., 2008), the reduced hippocampal ChAT activity following seizures may reflect a depletion of the cholinergic system as a result of a seizure-induced upregulation of neurogenesis in prenatally choline deficient rats that is comparable to control-fed rats.

Prenatal Choline's Effects on Adult Neurogenesis: Implications and Future Directions

The current dissertation research employed a variety of manipulations across experiments to investigate the effects of altered prenatal choline availability on properties of adult neurogenesis (basal and reactive) in the intact and injured brain. Table 2 presents a summary of these findings across all five experiments, in addition to relevant findings from a few additional previous studies, to highlight the differential effects of prenatal choline supplementation and deficiency on 1) basal levels of hippocampal neurogenesis across age, 2) reactive hippocampal neurogenesis in response to experience and seizures, and 3) basal levels of SVZ proliferation and olfactory bulb neurogenesis compared to control-fed rats. Also included within this summary are the effects of prenatal choline supplementation and deficiency on spatial learning and memory ability and expression levels of various neurotrophic and growth factors, as examined in the current experiments and available behavioral findings from a few previous studies.

Several noteworthy patterns emerge from these findings as a whole. First, the ability to detect an enhancement of baseline adult hippocampal neurogenesis with prenatal choline supplementation, given the techniques employed in the current and previous studies, does not emerge until at least 5 months of age (Experiment 5). This may

Condition	Prenatally Choline Supplemented vs. Controls			Prenatally Choline Deficient vs. Controls		
Across Age	Hippocampal Neurogenesis	Growth Factor	Learning & Memory	Hippocampal Neurogenesis	Growth Factor	Learning & Memory
2-2.5 mo (m) (Exp. 3 & 4)	n.e. BrdU+ n.e. DCX+	(+) BDNF (+) NGF (+) IGF-1 n.e. FGF2 n.e. NT-3	(+) Memory RAM m & f (Meck et al., 2008)	n.e. BrdU+ n.e. DCX+	n.e. BDNF n.e. NGF (-) IGF-1 (+) FGF-2 n.e. NT-3	(+) Memory RAM m & f (Meck & Williams, 2003)
5 mo (m) (Exp. 5)	(+) DCX+	(+) BDNF	n.e. Learning & Mernory MVVM (+) Mernory RAM, m (Meck & Williams, 1999)	N.A.	N.A.	(-) Memory RAM, when trials are massed, m (Meck & Williams, 1999)
7-9 mo (f) (Exp. 1)	(+) BrdU+ (+) Type-1 cells (+) Mitotic Sox-2+ cells n.e. BrdU+/NeuN+ (+) DCX+ (Glenn et al. 2007)	(+) BDNF (+) NGF	(+) Memory DMTP	n.e. BrdU+ n.e. stem cell phenotype n.e. Sox-2+mitotic activity (-) BrdU+/NeuN+	(+) BDNF n.e. NGF	n.e. Memory DMTP
16 mo (m) (Wong-Goodrich et al., 2008)	(+) BrdU+	(+) IGF-1	(+) Memory DMTP	N.A.	N.A.	n.e. Memory RAM 14.5 mo, m & f (Meck & Williams, 2003)
25-26.5 mo (m & f)	(+) BrdU+ (Glenn et al. 2008)	(+) VEGF (+) NT-3 (Glenn et al. 2008)	(+) Memory RAM (Meck et al., 2008)	N.A.	N.A.	n.e. Memory RAM (Meck & Williams, 2003)

Table 2. Summary of Findings on the Effects of Prenatal Choline Supplementationand Deficiency on Basal and Reactive Adult Neurogenesis, Hippocampal GrowthFactor Expression, and Spatial Learning and Memory.

Continued on next page

Condition	Prenatally Choline Supplemented vs. Controls			Prenatally Choline Deficient vs. Controls		
Following Enrichment	Hippocampal Neurogenesis	Growth Factor	Learning & Memory	Hippocampal Neurogenesis	Growth Factor	Learning & Memory
Spatial Exploration on RAM (Glenn et al., 2007)	n.e. BrdU+ n.e. DCX+	N.A.	N.A.	n.e. BrdU+ n.e. DCX+	N.A.	N.A.
Following Seizures	Hippocampal Neurogenesis	Growth Factor	Learning & Memory	Hippocampal Neurogenesis	Growth Factor	Learning & Memory
Short-term (Exp. 3 & 4)	(-) Brd∪+ n.e. DCX+	(-) BDNF (-) IGF-1 n.e.FGF-2	(+) Learning & Memory MWVM (Yang et al., 2000; Holmes et al., 2002)	n.e. BrdU+ n.e. DCX+	n.e. BDNF n.e. IGF-1 (-) FGF-2	n.e. Learning & Memory MWVM (Yang et al., 2000; Holmes et al., 2002)
Long-term (Exp. 5)	(+) BrdU+ (+) DCX+	n.e.BDNF	(+) Learning & Memory MVVM	N.A.	N.A.	N.A.
Other Neurogenic Regions	Neurogenesis	Growth Factor		Neurogenesis	Growth Factor	
SVZ (Exp. 2)	n.e. BrdU+	N.A.		n.e. BrdU+	N.A.	
Olfactory Bulb (Exp. 2)	(+) BrdU+ n.e. BrdU+/NeuN+	n.e. BDNF n.e. NGF		n.e. BrdU+ n.e. BrdU+/NeuN+	n.e. BDNF n.e. NGF	

Table 2 Cont.

BrdU+, bromdeoxyuridine-immunopositive cells. DCX, doublecortin-immunopositive cells. DMTP, delayed matching-to-place task. MWM, Morris water maze. RAM, radial arm maze. m, male. f, female. n.e., no effect. (+), increases. (-) decreases. N.A., not applicable.

be due to a high baseline rate of neurogenesis in the 2-month-old rat (Kuhn et al., 1996; Rao et al., 2006) used in Experiment 3, which may (in addition to the 10-day BrdU regimen, which captured a considerably large population of dividing cells) mask any effects of prenatal choline. Enhanced hippocampal trophic support is, however, detected in very young adulthood (2.5 months, Experiment 3; Table 2), which suggests that prenatally choline supplemented animals possess a hippocampal microenvironment conducive for enhanced neurogenesis even at an early young adult age when neurogenesis is already quite high. With age, however, adult hippocampal neurogenesis declines significantly in the adult rat and mouse (Seki and Arai, 1995; Kuhn et al., 1996; Kempermann et al., 1998; Seki, 2002; Bizon and Gallagher, 2003; Rao et al., 2005; Rao et al., 2006). In the adult rat, numbers of DCX+ neurons in the dentate gyrus remain stable at 4 to 7.5 months of age with the first major decline occurring between 7 and 9.5 months (Rao et al., 2006). Thus, a significant increase compared to control-fed rats in basal levels of adult hippocampal neurogenesis can be detected in prenatally choline supplemented rats prior to the first major period of age-related decline in neurogenesis, and then persists throughout young, middle-, and late adulthood (Table 2). This persistent increase in adult hippocampal neurogenesis throughout life may contribute to lifelong improvements in hippocampal memory, as prenatal choline supplemented rats compared to control-fed rats do not show age-related (up to 27.5 months) memory decline assessed by performance on a hippocampal-dependent radial-arm maze task (Meck and Williams, 2003; Meck et al., 2008). Others have similarly reported improvements in spatial learning and memory in aged mice and rats after increasing adult hippocampal neurogenesis with

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environmental enrichment or physical exercise (Kempermann et al., 1998; van Praag et al., 2005; Kim et al., 2010).

Another major pattern that emerges from these findings is that altered prenatal choline availability does not modulate adult neurogenesis in a dose-dependent manner or with regional specificity. While prenatal choline supplementation enhances basal levels of adult neurogenesis/newly divided cells in both the dentate gyrus and the olfactory bulb compared to control-fed animals, prenatal choline deficiency had minimal to no effect in both regions (Table 2). The only evidence for prenatal choline deficiency potentially having an effect on basal levels of adult neurogenesis was on slightly reducing the proportion of newly divided cells in the dentate gyrus that expressed a marker of neuronal maturation (Experiment 1), whereas prenatal choline supplementation had robust effects on proliferation and no effect on the rate of neuronal differentiation of newborn cells in the dentate (Experiment 1 and 5). Thus, supplementation and deficiency of choline in utero appears to affect different features of hippocampal neurogenesis in the adult brain with different degrees of magnitude. Perhaps this could be explained by a minimal to no effect of prenatal choline deficiency versus a robust and reliable effect of prenatal choline supplementation on hippocampal expression of various neurotrophic/growth factors (Table 2), or diet-related alterations in cholinergic function (as previously discussed) both of which are also not affected by prenatal choline availability in a dose-dependent manner per se. Or, the effects of choline deprivation lead to long-lasting adaptations that prepared the organism for living in a choline-deficient environment and are not simply a mirror image of the effects of prenatal choline supplementation.

Prenatal choline supplementation and deficiency also appear to exert differential effects on reactive hippocampal neurogenesis (Table 2). In response to maze exploration/enrichment, prenatally choline deficient rats do not upregulate hippocampal neurogenesis (Glenn et al., 2007), perhaps because prenatally choline deficient rats are unable to respond to neurogenic factors that are crucial for reactive neurogenesis. For example, hippocampal BDNF is both upregulated following enrichment and required for enrichment-induced neurogenesis (Rossi et al., 2006). Recent work has also demonstrated that neural stem cells and newborn neurons within the adult dentate gyrus express TrkB receptors and thus have the ability to respond to neurotrophic signals (Donovan et al., 2008). It is possible that prenatally choline deficient rats might fail to upregulate neurotrophic signals and/or lack the appropriate machinery to respond to these signals in response to experience. Experiment 1 began to address these issues, but the enrichment manipulation was not sufficient to produce any brain or behavior changes even in control-fed rats. Thus, it remains to be determined whether prenatal choline availability alters expression levels of hippocampal neurotrophic factors (and their high-affinity receptors) in response to environmental enriching experiences. However, prenatal choline deficient rats do show robust increases in dentate proliferation/neurogenesis and a number of neurotrophic/growth factors in response to seizures (Experiment 4), which suggests that the effects of prenatal choline deficiency on reactive neurogenesis in the adult hippocampus likely operates via different mechanisms depending upon the type of neurogenic stimuli.

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Exploration/enrichment also does not add to the increase in the number of BrdUand DCX-labeled cells observed in prenatally choline supplemented rats (Glenn et al., 2007). However, unlike deficient rats, supplemented rats have increased basal neurogenesis in the absence of exposure to an enriching experience. Notably, enriched control-fed rats upregulated to levels comparable to that of basal levels of prenatally choline supplemented rats (Glenn et al., 2007), suggesting that supplemented rats are likely operating at a ceiling. There may be a balance of plasticity in the hippocampus that prevents too much proliferation that may be disruptive to normal function, though the internal mechanisms that regulate this balance are clearly vulnerable to factors that compromise plasticity, like aging and epilepsy (i.e., months after initial status epilepticus). As suggested in Figure 52, prenatal choline supplementation may narrow the range of expression levels of adult hippocampal neurogenesis, shifting animals toward more favorable levels of neurogenesis needed for the hippocampal network to facilitate optimal cognitive function. Within this framework, prenatal choline supplementation without enrichment may produce an optimal level of hippocampal neurogenesis in supplemented rats such that when rats are already producing a near optimal number of new cells in the hippocampus, it is difficult to drive neurogenesis higher with environmental stimulation. As such, prenatally choline supplemented rats have enhanced memory, much like that of young, enriched, and/or physically exercised animals (Figure 52).

Also suggested by the model (Figure 52) is that prenatal choline supplementation may also work to restore an appropriate balance under conditions where abnormally high



Figure 52. Modulation of Adult Hippocampal Neurogenesis by Prenatal Choline Supplementation. The adult hippocampus regulates the amount of neurogenesis it expresses, negotiating optimal levels needed for the hippocampal network to facilitate optimal cognitive function. This balance, however, is vulnerable to factors that can either compromise levels of neurogenesis (aging, epilepsy) or stimulate disruptively high levels of neurogenesis (shortly after seizures). Similar to youth, enrichment, and physical exercise, prenatal choline supplementation enhances basal neurogenesis and cognitive function, and may narrow the range of expression levels of adult neurogenesis across contexts, shifting animals toward more optimal levels of neurogenesis for enhanced cognitive function. and potentially disruptive levels of dentate cell proliferation/ neurogenesis occur, like in response to prolonged seizures (Bengzon et al., 1997; Parent et al., 1997; Scharfman et al., 2002a), or conditions where neurogenesis levels are severely compromised, like in aging (Kuhn et al., 1996; Rao et al., 2006) and months after seizures (Hattiangady et al., 2004; Hattiangady and Shetty, 2010). Indeed, prenatally choline supplemented rats are characterized by: 1) increased basal neurogenesis (Table 2) and enhanced memory (Meck and Williams, 2003; Meck et al., 2008) throughout young, middle-, and late adulthood, 2) attenuated seizure-induced dentate cell proliferation and hippocampal growth factor expression (Experiment 3) and protection from learning and memory deficits shortly after seizures (Yang et al., 2000; Holmes et al., 2002), and 3) rescued levels of neurogenesis and recovery of spatial learning and memory function months after seizures when neurogenesis is diminished (Experiment 5). Although correlative, these cumulative findings highlight a role for adult neurogenesis as one potential mechanism for the lifelong memory-enhancing and neuroprotective effects of prenatal choline supplementation on behavior. The challenge for current researchers is to determine the optimal circumstances to enhance hippocampal neurogenesis for optimal cognitive function. Prenatal choline availability may offer one useful approach to address this issue.

Alternately, increased adult hippocampal neurogenesis by prenatal choline supplementation may make no direct contribution to cognitive function and may merely be a consequence of changes in the hippocampal microenvironment (i.e., increased expression of neurotrophic/growth factors) that support other types of hippocampal plasticity important for learning and memory. Indeed, prenatal choline supplementation

also enhances other features of adult hippocampal plasticity, such as LTP (Pyapali et al., 1998; Jones et al., 1999), NMDA receptor-mediated neurotransmission (Montova and Swartzwelder, 2000), dendritic spine density (Meck et al., 2008), and MAPK and CREB activity (Mellott et al., 2004). Thus, an increase in adult hippocampal neurogenesis may be just one of many changes in adult hippocampal plasticity elicited by prenatal choline supplementation. However, as discussed in the General Introduction, a review of the literature provides evidence that adult hippocampal neurogenesis is important for aspects of memory, and particularly under conditions where demands for plasticity are increased. Similarly, the current dissertation research supports the notion that prenatal choline supplementation benefits brain and behavior especially under conditions that place demands (e.g., cognitive task demands, hippocampal aging, seizures; see Table 2 summary) on the animal. For example, Experiment 1 revealed that prenatally choline supplemented animals showed enhanced memory function during the long, but not short retention delay or spatial acquisition of the DMTP task. Experiment 5 showed that while saline-treated supplemented rats performed comparably to that of saline-treated controlfed rats on a minimally challenging standard water maze task, the benefits of prenatal choline supplementation were clearly evident for KA-treated rats who exhibited seizureinduced learning and memory deficits.

That the behavioral benefits of prenatal choline supplementation appear to be more robust for aspects of memory as opposed to learning (Experiments 1 and 5) is consistent with ablation studies using hippocampal tasks that have demonstrated a clear role for adult hippocampal neurogenesis in the expression of memory, and not learning (Shors et al., 2001; Snyder et al., 2005; Winocur et al., 2006; Kee et al., 2007; Deng et al., 2009; Kitamura et al., 2009; Trouche et al., 2009). Thus, it is predicted that enhanced adult hippocampal neurogenesis by prenatal choline supplementation likely makes some contribution to improvements in memory on hippocampal-mediated tasks. Future studies that include an ablation approach to directly manipulate levels of neurogenesis in prenatally supplemented and control-fed rats are needed to directly test this hypothesis. Using the anti-mitotic agent methylazoxymethanol acetate (MAM) to reduce neuronal proliferation may be a useful approach because MAM action is transient, which allows for a relatively quick restoration of neurogenesis (e.g., 3 weeks, Shors et al., 2001), and can be potentially titrated to a smaller dose to reduce levels of neurogenesis by a desired percentage. Thus, one could conceivably reduce neurogenesis levels in prenatally choline supplemented rats to levels comparable to that of control-fed rats via MAM, and then test whether or not prenatally choline supplemented rats still exhibited a memory enhancement. If enhanced neurogenesis made a significant contribution to the improvements in memory function in supplemented rats (i.e., if the memory enhancement was indeed abrogated by MAM treatment), one could then behaviorally retest the animals after allowing neurogenesis levels to recover to assess whether an enhancement of memory could be restored.

Conclusions

The present collection of studies demonstrates that altered prenatal choline availability modulates properties of adult hippocampal neurogenesis in the intact and injured brain that may ultimately contribute to changes in learning and memory function. Although correlative, the current findings stimulate new avenues of research investigating the functional contribution of increased neurogenesis (throughout life and in diseased states) by prenatal choline supplementation that may be potentially addressed using ablation approaches. The results of this dissertation also revealed that prenatal choline's effects on adult neurogenesis are not specific to the hippocampus, although additional studies are needed to further characterize the effects of altered prenatal choline availability on olfactory bulb neurogenesis, including whether reactive neurogenesis in the adult olfactory bulb (e.g., via enriched odor exposure, Rochefort et al., 2002) is similarly modulated as it is in the adult hippocampus by prenatal choline availability, and whether more newborn olfactory bulb neurons confer any functional benefit for prenatally choline supplemented animals.

The current findings from this dissertation research may also have important translational and clinical implications, as the estimated intake of choline by pregnant women is frequently less than half the amount of the normal human consumption amount of 425-550 mg/day (Zeisel, 2004; Zeisel, 2006). While the experiments in this dissertation examined only the effects of choline supplementation during prenatal development, some reported memory-enhancing and neuroprotective actions of choline appear to be postnatal (Meck and Williams, 2003; Thomas et al., 2004; Thomas et al., 2007; Meck et al., 2008). Thus, human infants might also benefit from added choline to formula or choline supplementation to mother's diet.

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Honors and Awards

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- Annie Laurie Aiken Fellowship in Neuroscience, National Science Foundation, Duke University (2006)
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