UNIVERSITY OF CALIFORNIA, SAN DIEGO

Neural Correlates of Age-Related Memory Decline and Sensitivity to BDNF Gene Delivery

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Neurosciences

by

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2008

The dissertation of Tonya R. Mead is approved, and it is acceptable in quality and form for publication on microfilm:

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2008

DEDICATION

To my beloved husband, who makes the impossible possible and is a source of joy in my every day.

To my mom, who has been my best friend since the day we met.

To my dad, who has taught me to view every challenge as a new adventure.

To Ted and Judy, who have provided endless support and encouragement throughout this process.

To Myles, the big brother I have always wanted, and a dear friend.

To all of my family and friends, who allow me to be myself and love me anyway. Without you this thesis would not exist.

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ACKNOWLEDGEMENTS

I would like to thank my advisor Mark Tuszynski, a mentor and friend, whose guidance was critical for the success of this thesis. I would also like to thank my committee members, Andrea Chiba, Anirvan Ghosh, Edward Koo, and Eliezer Masliah for their invaluable advice. I owe a particular thank you to Dr. Masliah who collaborated on work described in chapter 2. The members of the Tuszynski lab, past and present, are dear friends, and to them I owe a great debt of gratitude for their encouragement and support, both scientific and moral. In particular, I would like to acknowledge James Conner and Armin Blesch who collaborated on the work described in this thesis. I am also grateful for the help of Ling Wang, Ronald Torres, Deborah Otero, Karin Loew, Alan Nagahara, David Merrill, and Brendan Brinkman.

Chapter 2, in full, is in preparation for publication. Tonya Mead and Mark Tuszynski are the authors of this manuscript. The dissertation author was the primary investigator and first author of this paper.

Chapter 3, in full, is in preparation for publication. Tonya Mead, Eliezer Masliah, Armin Blesch, and Mark Tuszynski are the authors of this manuscript. The dissertation author was the primary investigator and first author of this paper.

The data in Chapter 4 are the result of the combined efforts of Tonya Mead, James Conner, and Mark Tuszynski.

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ABSTRACT OF THE DISSERTATION

Neural Correlates of Age-Related Memory Decline and

Sensitivity to BDNF Gene Delivery

by

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Doctor of Philosophy in Neurosciences

University of California, San Diego, 2008

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Spatial memory is susceptible to age-related impairment and relies on intact function of the entorhinal cortex. In this thesis, we examine the hypothesis that normal aging is characterized by structural degeneration of entorhinal neurons, and predict that these changes are reversible through the administration of brain-derived neurotrophic factor (BDNF).

In the medial entorhinal cortex, cell filling and spine density analysis of perforant path-projecting neurons revealed that aging results in decreased excitatory

input to these neurons. Aged rats exhibited a significant reduction in the density of basilar (18.2%) and apical (16.0%) dendritic spines. Mushroom spines, the most active and stable spine type, exhibited a disproportionate decline with aging. We also found decreased density of the presynaptic marker synaptophysin in the dentate molecular layer of aged rats, suggesting that aged neurons of the medial entorhinal cortex provide fewer inputs to the hippocampus.

Age-related changes in neuron structure may be sensitive to brain-derived neurotrophic factor, a protein that influences adult neuron plasticity. To test this hypothesis, we delivered BDNF to the medial entorhinal cortex through lentiviral vector administration. The expression of BDNF, but not NGF or LacZ, reversed the age-related decline in spine density and increased the density of three spine types. Furthermore, the expression of BDNF, but not EGFP, in the entorhinal cortex increased synaptophysin density in the aged rat dentate gyrus.

BDNF also influences synaptic function. Based on previous experiments, we predicted that BDNF lentiviral vector delivery to the aged entorhinal cortex would increase long-term potentiation (LTP) in the dentate hilus following tetanic stimulation of the perforant path. On the contrary, we found that chronic BDNF administration significantly reduced hippocampal LTP in comparison with the responses of aged control rats or young subjects. Possible mechanisms are discussed.

Collectively, these results indicate that normal aging leads to structural degeneration of perforant path-projecting neurons in the medial entorhinal cortex, and that the changes occur in plastic regions: presynaptic and postsynaptic terminals. Furthermore, lentivirus-mediated BDNF delivery to the entorhinal cortex reverses age-related structural degeneration of entorhinal neurons and modifies the function of perforant path–dentate granule cell synapses.

I

Introduction

Although memory loss that occurs with normal aging is less debilitating than that of Alzheimer's disease or dementia, it can still have a large influence on an individual's health and independence. By investigating neural mechanisms underlying age-related cognitive decline and testing methods for reversing these changes, we hope to eventually improve of the quality of later life.

The types of memory changes that are commonly expressed with normal aging point to functional changes in the prefrontal cortex and hippocampal formation (reviewed in Hedden and Gabrieli, 2004). Although substantial effort has been made to understand the role of the hippocampal formation in age-related memory decline, to date, our understanding of age-related changes in this system remains incomplete. In this dissertation, we have focused our investigation on the entorhinal cortex, a component of the hippocampal formation and a region important for spatial memory encoding. We have tested the hypothesis that perforant path-projecting neurons in the medial entorhinal cortex undergo structural degeneration with advancing age.

Additionally, we investigated whether these structural changes are reversible through increasing neurotrophic support to the region.

The neurons of the medial entorhinal cortex naturally express brain-derived neurotrophic factor (BDNF), a member of the classic neurotrophin family, and TrkB, its high-affinity receptor. BDNF promotes neuroplasticity in the adult brain, and may have the ability to increase the structural and functional plasticity of neurons in the aged brain. In the second part of this dissertation, we tested the hypothesis that BDNF can enhance the function and reverse the structural deterioration of aged neurons in the medial entorhinal cortex.

The following introduction provides the conceptual framework for our hypotheses. The chapter begins with a review of the cognitive changes found with normal aging. It then continues with evidence that the entorhinal cortex is important for spatial memory, one form of age-altered memory, and, additionally, that the entorhinal cortex is vulnerable to age-related degeneration. The next section describes structural changes identified in the aged brain, followed by a review of BDNF and its effects on neuroplasticity. This chapter will conclude with the general hypothesis and specific aims of the thesis.

A. Memory Decline with Normal Aging

Cognitive decline is a common feature of advancing age. In a recent epidemiology study, only 45% of individuals over the age of 84 were cognitively normal (Unverzagt et al., 2001). The changes in memory that occur with normal aging, absent of disease or dementia, progress gradually. In fact, measurable decreases in some cognitive functions may occur as early as the second decade in human subjects (Park et al., 2002), but they are not noticeable until much later. Despite some measurable cognitive changes, most forms of memory are preserved into middle age or later.

Memory is not a unitary function, and some forms of memory are more vulnerable to age-related impairment. Memory functions may be grouped into five clinically relevant categories: procedural memory, priming, semantic memory, working memory, and episodic memory (reviewed in Budson and Price, 2005; Tulving, 1987; Tulving et al., 1990; Nilsson, 2003). Procedural memory refers to learned behavioral or cognitive skills that can be accessed and used at an automatic level (e.g., riding a bike). This form of implicit memory is unaffected by normal aging (Churchill et al., 2003). Priming, another form of implicit memory, is also maintained with normal aging (Nilsson, 2003; Fleischman et al., 2004). Priming is a function of the visual cortex (Squire et al., 1992; Kroll et al., 2003) in which previous exposure to

a stimulus increases the speed or accuracy of performance on a test without requiring conscious recall. The third category, semantic memory, encompasses our collective knowledge of words, facts, and concepts. The effect of normal aging on semantic memory varies between studies. While some studies indicate life-long increases in vocabulary (Park et al., 2002), others show that verbal memory peaks between the ages of 50 to 60 years, and then declines (Schaie, 1994; Nilsson, 2003). Working memory and episodic memory are the types most affected by normal aging. Working memory refers to the ability to maintain and process information in our conscious minds over a relatively short period of time, and the prefrontal cortex plays a complex, but important role in this process (Müller and Knight, 2006). Age-related deficits in working memory increase with increasing difficulty of the task (Babcock and Salthouse, 1990; Gazzaley et al., 2007). Episodic memory describes the recollection of our own personal experiences, and often undergoes the most dramatic decrease with advancing age (Park et al., 2002; Nilsson, 2003). Many brain regions are important for encoding episodic memory, but the hippocampal formation is of particular importance (Squire and Zola-Morgan, 1991).

The age-related declines in episodic and working memory predict changes to the hippocampal formation and prefrontal cortex. Indeed, the volumes of the hippocampus and lateral prefrontal cortex decrease with advancing age, whereas several other regions, including the inferior parietal cortex and anterior cingulate

gyrus, remain unchanged (Raz et al., 2004). Notably, longitudinal data correlate episodic memory decline with decreased hippocampal volume (Kramer et al., 2007). Changes in white matter integrity throughout the brain may also undermine cognitive function in healthy aged individuals (Guttmann et al., 1998; Gunning-Dixon and Raz, 2000; Head et al., 2004). As I will highlight in later chapters, these structural changes are not likely the result of gross cell loss, rather they may reflect age-related decreases in axonal projections throughout the brain and decreased synaptic contacts within the regions governing episodic and working memory.

B. Spatial Memory and the Entorhinal Cortex

Spatial memory is a form of episodic memory that is particularly susceptible to age-related decline (Perlmutter et al., 1981; Sharps and Gollin, 1987; Uttl and Graf, 1993). Spatial memory provides us with cognitive maps of physical space that we use to recall information about the location and orientation of objects in a particular environment. Spatial memory impairment with normal aging is not limited to humans; aged rats also develop spatial learning deficits (Barnes, 1979; Gage et al., 1984; Gallagher, 1993). In male Fischer 344 rats, the focus of our studies, the prevalence of spatial memory decline is very high with significant impairment in at least 89% of the population by 23 months of age (Chapter 2).

As with other types of episodic memory, spatial memory is a function of the hippocampal formation. This complex consists of the hippocampus proper (dentate gyrus, CA1, CA2, and CA3), subiculum, and entorhinal cortex (EC). It is structured primarily as a unidirectional loop that begins and ends in the entorhinal cortex (Witter et al., 2000). The perforant path provides the major source of excitatory input to the hippocampus. It consists of EC layer II neurons that project to dentate granule cells. Some EC layer II neurons also project to CA3, and EC layer III neurons project to CA1. From the entry of the perforant path into the hippocampus proper, the primary excitatory loop projects in the following order: DG, CA3, CA1, and subiculum. The loop is completed by projections from the subiculum to layers V and VI of the entorhinal cortex. This places the entorhinal cortex in the unique position of regulating the excitatory connections between neocortex and the hippocampus.

Studies of individuals with extensive damage to the hippocampal formation provided the first insights into the importance of this region for episodic memory, including its role in the formation and maintenance of recent spatial memory (Scoville and Milner, 1957). Remote spatial memories, those that were formed long before the damage occurred, are likely stored elsewhere in the brain and retrieved by a separate mechanism (Teng and Squire, 1999; Rosenbaum et al., 2000). Although lesion studies (O'Keefe et al., 1975; Morris et al., 1982; Sutherland et al., 1983; Gallagher and Holland, 1992) and place field electrophysiology (O'Keefe and Dostrovsky, 1971;

McNaughton et al., 1983; Muller et al., 1987) firmly established the importance of the hippocampus proper for spatial memory function decades ago, the importance of the entorhinal cortex in spatial memory encoding remained unclear (reviewed in Aggleton et al., 2000; Burwell et al., 2004). That is, until a recent study demonstrated that fiber-sparing lesions of the dorsolateral band of the medial EC disrupted recently acquired spatial memory and impaired further spatial memory acquisition (Steffanach et al., 2005). Lesions of the ventromedial band had no affect on spatial memory. The authors suggest that the inconsistent results from previous lesion studies may be attributed to partial sparing of the dorsolateral band.

The neurons in the dorsal part of the medial EC are well positioned for a role in spatial memory function. The neurons in layer II of this region receive the majority of visuospatial input to the hippocampal formation from the postrhinal cortex (Burwell, 2000), and also receive afferents directly from the occipital and parietal cortices (Kerr et al., 2007). The layer II neurons in the dorsomedial entorhinal cortex form the primary excitatory projection to the septal (dorsal) part of the hippocampus (Dolorfo and Amaral, 1998; Burwell, 2000), which has the largest place cell population within the hippocampus (Jung et al., 1994) and has a greater influence on spatial memory (Moser et al., 1995; Moser and Moser, 1998) than the temporal pole.

A landmark electrophysiological study provided new insight into the mechanisms for encoding spatial information in the hippocampal formation. This

study revealed the presence of grid cells within the dorsal portion of the medial entorhinal cortex (Fyhn et al., 2004), and a later study determined that these cells exist in all principle cell layers of this region (Sargolini et al., 2006). The name "grid cells" is based on the firing patterns of the cells as rats move freely around an enclosed area; the cells produce multipeaked firing fields in a tessellating grid-like pattern (Fyhn et al., 2004). The spatial response grid originates in the medial entorhinal cortex and is modified by, but not dependent on, hippocampal input (Fyhn et al., 2004). The location of the rat and its trajectory can be precisely reconstructed from the ensemble activity of a small number of grid cells. In addition to providing a mechanism for spatial navigation, grid cells in the entorhinal cortex may play a role in spatial memory formation. Grid cell response is stable across multiple trials in the same test environment (Fyhn et al., 2004; 2007), rotates in response to cue rotation (Hafting et al., 2005; Sargolini et al., 2006), and shifts in response to a new environment, predicting global remapping of place cell activity within the hippocampus (Fyhn et al., 2007). These studies provide strong evidence that the entorhinal cortex is integral to spatial memory encoding. Therefore, the effect of aging on the entorhinal cortex warrants further investigation.

C. Entorhinal Cortex and Normal Aging

Few studies have investigated changes in the entorhinal cortex that result from normal aging, though it has been the target of much investigation in studies of Alzheimer's disease. The entorhinal cortex shows measurable loss of volume even in early Alzheimer's disease (Juottonen et al., 1998) and suffers the greatest damage of any cortical region throughout the course of the disease (Van Hoesen et al., 1991; 2000). Decreased volume of the entorhinal cortex is clear even with mild Alzheimer's disease (Juottonen et al., 1998; de Toledo-Morrell et al., 2000; Kordower et al., 2001). In patients with mild cognitive impairment, the volume of the entorhinal cortex helps predict conversion to Alzheimer's disease (de Toledo-Morrell et al., 2004; Devanand et al., 2007). Neurofibrillary tangles are common in layer II of the entorhinal cortex (Hyman et al., 1984; Braak and Braak, 1985; Goméz-Isla et al., 1996), and spread with the progression of the disease (Van Hoesen et al., 2000). Most telling is the extensive neuron loss that occurs in this region. The neurons in layers II and IV of the entorhinal cortex are the first to show measurable decreases in Alzheimer's disease (Hyman et al., 1984; Goméz-Isla et al., 1996), and the loss of layer II neurons correlates with declines in declarative memory (Kordower et al., 2001). Extensive cell loss in the entorhinal cortex is characteristic of more advanced stages of Alzheimer's disease (Goméz-Isla et al., 1996).

Like Alzheimer's disease, normal aging alters the integrity of the entorhinal cortex, but the changes are less dramatic. Normal aging leads to a small decrease in the volume of the entorhinal cortex (de Toledo-Morrell et al., 2000; Rodrigue and Raz, 2004). Although the change is mild compared to that found in Alzheimer's disease, a higher rate of EC shrinkage is predictive of declining memory performance (Rodrigue and Raz, 2004). Neuron loss does not contribute to the decreased EC volume in healthy aging. Early studies suggested rampant neuron loss in the aged brain (Brody, 1955; Shefer, 1973; Ball, 1977), but their results later proved to be a consequence of differential tissue shrinkage in young and aged samples (Haug, 1985; reviewed in Peters et al., 1998). More recent stereological analyses have determined that age-related neuronal degeneration affects very few cortical and subcortical regions (Smith et al., 2004; reviewed in Peters et al., 1998) and does not occur in the entorhinal cortex (Amaral, 1993; Gazzaley et al., 1997; Merrill et al., 2000; Merrill et al., 2001; Rapp et al., 2002). Similar studies in the human hippocampus provide conflicting results (reviewed in Peters et al., 1998), but studies in aged monkeys and rats consistently show no loss of hippocampal neurons (Amaral, 1993; Rapp and Gallagher, 1996; Rasmussen et al., 1996). Neuron number in the hippocampus and entorhinal cortex is also preserved in rats with age-related spatial memory impairment (Rapp and Gallagher, 1996; Rasmussen et al., 1996; Merrill et al., 2001; Rapp et al., 2002).

Although neuron loss in the entorhinal cortex cannot account for the spatial memory decline in normal aging, the small decrease in EC volume suggests these neurons may experience structural deterioration. An age-related loss of axospinous synapses in the middle molecular layer (MML) of the dentate gyrus is consistent with decreased synaptic input from the medial entorhinal cortex to the dentate granule cells (Geinisman et al., 1992). This possibility is further supported by reduced MML volume in aged rats (Rapp et al., 1999). These changes in a terminal zone of layer II entorhinal neurons, coupled with the early and dramatic loss of EC layer II neurons in Alzheimer's disease, suggests these neurons may be vulnerable to age-related cellular insults. Therefore, in this thesis, we have investigated the layer II neurons of the medial entorhinal cortex for age-related structural changes.

D. Normal Aging and Changes in Neuron Structure

Although neuron loss is not a widespread feature of normal aging, changes in neuron structure are common. These more modest changes can have a large influence on cognition by disrupting the normal communication between neurons.

Early studies of age-related changes in dendrite branching suggested extensive deterioration of neuronal arbors in the entorhinal cortex and hippocampus (Scheibel et al., 1976; Scheibel, 1979), but improved screening for dementia and use of

stereological controls led to re-evaluation of these changes (reviewed in Burke and Barnes, 2006). The extent of dendritic arborization remains stable in many brain areas with normal aging. In the human hippocampus, neurons in CA1 (Hanks and Flood, 1991), CA2-3 (Flood et al., 1987), and layer III of the subiculum (Flood, 1991) show net stability of their dendritic arbors. Dendrite extent, dendrite length and degree of dendritic branching, is also stable in the entorhinal cortex layer II neurons of aged rats (Coleman and Flood, 1991), but terminal branch extent increases in parahippocampal gyrus layer II neurons in aged humans (Buell and Coleman 1979; 1981). The human parahippocampal gyrus is a structure that includes the entorhinal cortex. Increased dendritic extent in aged subjects may be a form of neuroplasticity that compensates for decreased synaptic input. Similar age-related increases in dendritic arborization occur in the rat somatosensory cortex (Connor et al., 1982) and human dentate gyrus (Flood et al., 1985; 1987). The aged prefrontal and medial frontal cortices, on the other hand, show mild dendrite regression (Cupp and Uemura, 1980; Jacobs et al., 1997; Grill and Riddle, 2002). The region-specific dendritic changes associated with normal aging are fairly small and often limited to distal or terminal branches. Based on this evidence, dendritic regression does not greatly contribute to age-related cognitive decline.

The extensive literature on age-related decreases in synapses and postsynaptic spines suggests that loss or alterations of these plastic structures may form a primary mechanism for cognitive decline in normal aging. Age-related changes in synapses are

region specific, and frequently layer specific. For example, synapse density decreases with aging in layer II, but not layer IV, of the rat sensorimotor cortex (Brunso-Bechtold et al., 2000).

In the hippocampal formation, synapse number is maintained with advancing age in CA3 stratum lucidum (Poe et al., 2001), where dentate mossy fibers terminate, and in CA1 stratum radiatum (Geinisman et al., 2004), where CA3 projections synapse. Spine density measurements provide further support for a general sparing of the CA3-CA1 synapses. While the overall spine density of aged CA1 pyramidal neurons shows a mild decrease of 11 to 12% (Nunzi, 1987), the density of spines in the stratum radiatum remains unchanged (Markham et al., 2005). Although synapse number is maintained in the CA1 stratum radiatum with aging, a recent study suggests that decreased postsynaptic density area in of a subset of these synapses may contribute to age-related spatial memory impairment (Nicholson et al., 2004). The aging effects on the hippocampal intrinsic connections are relatively small in comparison to the age-related changes to afferent inputs from the entorhinal cortex.

The middle molecular layer of the dentate gyrus has shown the greatest age-related synaptic changes within the hippocampus. In two early studies, axospinous synapse number decreased by about 26% in aged rats (Bondareff and Geinisman, 1976; Geinisman and Bondareff, 1976). A later study that used unbiased stereology confirmed this change (Geinisman et al., 1992). It also determined that the axospinous

synapses in the inner molecular layer of the dentate gyrus decrease by about 24% with aging. This region receives input from commissural-associational fibers. Synaptophysin immunolabeling in the middle and inner molecular layers of the aged rat dentate gyrus provides support for these studies (Chapter 3). In both regions, the density of the synaptophysin label decreased in aged rats suggesting fewer presynaptic boutons in those layers.

Age-related decreases in synapses and spines can be substantial, approaching 50% in some brain regions in humans (Jacobs et al., 1997), and may produce the cognitive changes associated with normal aging. This is, perhaps, good news since there are many methods for increasing spine and synapse number, with increased neurotrophin exposure among the most effective methods. This thesis investigates whether brain-derived neurotrophic factor, a member of the classical neurotrophin family of proteins, is able to increase the spine and synaptic plasticity of aged neurons and reverse their age-related decline.

E. Additional Changes with Normal Aging

In addition to structural changes in aged neurons, changes in gene expression, cell signaling, and synaptic plasticity influence neuron function in the aged hippocampal formation, and may contribute to age-related cognitive impairment.

Changes in gene expression in the hippocampus with normal aging are wide-ranging. Age-related decrements have been found in genes involved in neuronal plasticity, including IGF-II and c-fos (Kitraki et al., 1993) and calcium homeostasis, including calbindin-D28k (Kishimoto et al., 1998). The expression of mRNA for GABA receptor subunits and proteins involved in neuroinflammation (e.g., IL-1β and TNF-α), on the other hand, exhibit age-associated increases (Ruano et al., 2000; Gavilán et al., 2007). Yet others, including the genes that encode AMPA receptor subunits, remain unaffected by normal aging (Ruano et al., 2000). One study, in particular, utilized microarray analysis of hippocampal tissue (from CA1) in behaviorally characterized, aged rats to investigate categorical trends in gene expression changes that related to both aging and cognitive performance (Kelly et al., 2005). The authors of this study identified increased expression of inflammatory markers and decreased expression of genes involved in energy metabolism and synaptic plasticity.

Changes in intracellular signaling with aging, particularly in the MAPK/ERK signaling pathway can alter synaptic plasticity. Intracellular signaling cascades are commonly activated by transmembrane receptors, such as TrkB, and are a source of information flow within neurons. These cascades are important for neuronal differentiation (reviewed in Kaplan and Miller, 2000; Patapoutian and Reichardt,

2001), survival (Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001) and plasticity (see Sheng and Kim, 2002 for review; Jourdain et al., 2003; Pilpel and Segal, 2004), among other activities. Aging has selective influences on TrkB-activated signaling cascades in the aged entorhinal cortex of cognitively impaired rats (Merrill, 2002). Constitutive activity of the MAPK/ERK pathway in aged-impaired rats was significantly reduced compared to young and aged-unimpaired rats as determined by Western blot analysis of phospho-ERK expression (Merrill, 2002). The PI-3K/Akt1 pathway, on the other hand, was unaffected by aging or cognitive decline. In a related study, the expression of Arc was measured in aged hippocampal neurons (Small et al., 2004). Arc, a transcription factor and immediate-early gene, is upregulated by TrkB activation and intracellular signaling (Ying et al., 2002), and is important for synaptic plasticity (Messaoudi et al., 2007). Small and colleagues measured the percent of cells expressing Arc mRNA in the dentate gyrus, CA3, and CA1, and determined that Arc expression is most susceptible to age-related decline in the dentate gyrus (2004).

Normal aging also produces changes in hippocampal synaptic plasticity (reviewed in Rosenzweig and Barnes, 2003); these are commonly measured as changes in the induction of long-term potentiation (LTP). In CA1, low-level, perithreshold stimulation has revealed age-related deficits in LTP induction (Deupree et al., 1991; Moore et al., 1993; Rosenzweig et al., 1997), whereas traditional high-frequency stimulation (HFS) protocols obscure these effects (Landfield et al., 1978;

Deupree et al., 1993; Moore et al., 1993; Diana et al., 1994a,b; Norris et al., 1996). Findings in the dentate gyrus have been less consistent. While several groups have found no age-related changes in LTP induction following high-frequency stimulation (Barnes, 1979; Diana et al., 1994a,b), another has reported impaired induction (Lynch and Voss, 1994; Mullany and Lynch, 1997; Kelly et al., 2000). As in the CA1, LTP induction deficits in the dentate gyrus were more apparent with a less robust stimulation. A protocol combining perithreshold stimulation of the perforant path with granule cell depolarization revealed an age-related increase in LTP induction threshold (Barnes et al., 2000). Together, these studies indicate that aging decreases the functional plasticity of hippocampal synapses, and that these effects can be found in both the dentate gyrus, where layer II entorhinal neurons form synapses, and in CA1.

Although the changes in neuron structure, gene expression, cell signaling, and synaptic plasticity in the aged hippocampal formation can be studied separately, they are not independent phenomena. Together, they provide a more complete picture of the mechanisms that underlie age-related cognitive impairment.

F. BDNF and Neuroplasticity

Brain-derived neurotrophic factor is a member of the neurotrophin family of proteins. Members of this family that are expressed in the mammalian brain include

nerve growth factor (NGF), the first discovered in the 1950s (Levi-Montalcini and Hamburger, 1951); neurotrophin-3 (NT-3); and neurotrophin 4/5 (NT-4/5). While all four of these neurotrophins bind to the low-affinity p75 neurotrophin receptor (p75NTR), the majority of their effects are initiated through high-affinity binding with receptor tyrosine kinases in the tropomyosin receptor kinase (Trk) family. The target of NGF is TrkA, BDNF and NT-4/5 activate TrkB, and NT-3 preferentially binds with TrkC. In the developing brain, these neurotrophins promote neuron survival (reviewed in Davies, 1994), enhance neuronal differentiation (Sieber-Blum, 1991; Ghosh and Greenberg, 1995; Li et al., 1995; Vicario-Abejón et al., 1995), initiate axon growth, and provide axon guidance (reviewed in Gillespie, 2003). In the adult brain, both BDNF and NGF significantly influence neuroplasticity.

BDNF and NGF promote neuroplasticity in the adult brain through separate mechanisms. This is due, in part, to the differing expression profiles of their high-affinity receptors. TrkA, the NGF receptor, is expressed by cholinergic neurons in the basal forebrain (Holtzman et al., 1992; Steininger et al., 1993; Sobreviela et al., 1994; Holtzman et al., 1995). These neurons send axonal projections throughout the cortex and hippocampus (Wenk et al., 1980; McKinney et al., 1983; Luiten et al., 1985; Gaykema et al., 1990), and TrkA receptors reside on their axon terminals (Rossi et al., 2002; Prakash et al., 2004). NGF-sensitive cholinergic inputs to hippocampus modulate synaptic plasticity and some aspects of behavior. In cortex, stimulation of

TrkA receptors increases the cholinergic activation of a small neural network and expands the cortical representation of a particular function (Prakash et al., 2004; reviewed in Prakash et al., 2005). Therefore, NGF indirectly modulates cortical neuroplasticity by increasing the activity of cholinergic afferents to cortical regions. In contrast, both BDNF and its receptor, TrkB, are expressed by glutamatergic neurons throughout adult cortex and hippocampus (Fryer et al., 1996; Yan et al., 1997a,b; Tokuyama et al., 1998). This enables BDNF to have a direct impact on the plasticity of these neurons.

BDNF enhances the synaptic activity of adult neurons through a variety of mechanisms. At presynaptic terminals, BDNF facilitates neurotransmitter release by increasing intracellular calcium concentration (Stoop and Poo, 1996; Li et al., 1998) and increasing the number of vesicles docked at the active zone (Tartaglia et al., 2001; Tyler and Pozzo-Miller, 2001). At postsynaptic terminals, BDNF enhances glutamate-evoked responses by increasing the NMDA-channel open probability (Levine et al., 1998).

The ability of BDNF to enhance the structural plasticity of adult neurons is particularly remarkable. In transgenic mice, a modest increase in endogenous hippocampal BDNF caused a significant increase in the length and complexity of the dentate granule cell dendritic arbors (Tolwani et al., 2002). Postsynaptic spines are also very responsive to BDNF. In vitro, BDNF administration increased the spine

density of Purkinje and hippocampal pyramidal neurons (Shimada et al., 1998; Tyler and Pozzo-Miller, 2001; Tyler and Pozzo-Miller, 2003). Later studies determined that the BDNF-induced increase in hippocampal spine density is dependent on the MAPK/ERK signal transduction pathway (Alonso et al., 2004) and modulated by cAMP (Ji et al., 2005). BDNF also modifies synapse morphology. On presynaptic terminals, BDNF increases the length of the active zone enabling a greater number of vesicles to dock there (Tyler and Pozzo-Miller, 2001). BDNF may also influence the structure and function of postsynaptic spines through its effect on presynaptic glutamate release. A recent study demonstrated that repetitive glutamate release at a synapse increases spine volume and enhances AMPA-receptor-mediated currents (Matsuzaki et al., 2004).

Because of its influence on synaptic plasticity in the hippocampal formation, changes in BDNF expression with age could affect the activity in this region, and impair cognitive function. Support for this hypothesis comes from a recent study in which selective deletion of BDNF in the dorsal hippocampus of adult transgenic mice resulted in impairment of spatial learning (Heldt et al., 2007). So far, two studies that measured BDNF expression in the aged hippocampus and entorhinal cortex have produced conflicting results. In one study, BDNF expression declined throughout the hippocampus and entorhinal cortex (excepting CA2) with advanced age in macaque monkeys (Hayashi et al., 2001). Research from our group, on the other hand, has

found no change in BDNF expression in either the hippocampus or entorhinal cortex of aged rats (Merrill, 2002). Nevertheless, a separate study from our lab has demonstrated amelioration of age-related spatial memory impairment following long-term BDNF infusion into the medial entorhinal cortex (Merrill, 2002). These data predict that, regardless of endogenous expression levels, increased BDNF expression in the aged entorhinal cortex can reverse the structural and functional correlates of age-related spatial memory decline.

G. Hypothesis and Specific Aims

In this thesis, we have investigated changes in the entorhinal cortex that may contribute to age-related spatial memory decline, and we have tested whether BDNF can reverse these changes. Specifically, this dissertation tests the hypothesis that: **Structural deterioration of perforant path-projecting neurons in the medial entorhinal cortex accompanies normal aging and is sensitive to BDNF gene delivery.**

The following specific aims will be addressed:

1) Examine age-related changes in postsynaptic spine density and structure on perforant path-projecting neurons of the rodent dorsomedial entorhinal cortex. Determine whether these changes suggest an age-related decrease in neuronal function.

2) Determine whether BDNF gene delivery to the dorsomedial entorhinal cortex in aged rats can reverse the age-related structural deterioration of these neurons. First, investigate changes in postsynaptic spine density and structure following BDNF, NGF, and LacZ lentiviral vector delivery to the dorsomedial entorhinal cortex. Second, examine changes in synaptophysin density in the dentate gyrus following BDNF or EGFP lentiviral vector delivery to the dorsomedial entorhinal cortex.

3) Investigate the hypothesis that BDNF gene delivery to the dorsomedial entorhinal cortex in aged rats increases the tetanus-induced potentiation of medial perforant path-dentate granule cell synapses.

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II

Age-Related Spine Density Decline and Preferential Loss of Large Spines in the Medial Entorhinal Cortex

A. Abstract

Dendritic spines play an essential role in cognition; changes in spine density and structure could undermine synaptic transmission and neural function. We investigated alterations in these measures as a function of age-related spatial memory impairment in layer II neurons of the entorhinal cortex in 23-month-old Fischer 344 rats. Aged rats demonstrated consistent impairment in spatial learning and memory compared to young subjects. We then injected a retrograde tracer into the dorsal hippocampus of young and aged subjects to specifically label hippocampus-projecting neurons in layer II of the entorhinal cortex. Retrogradely labeled cells were filled with lucifer yellow in slice preparations and subjected to detailed spine analysis. Aged rats exhibited a significant 18.2% decrease in basilar spine density ($P < 0.001$) and a 16.0% decrease in apical spine density ($P < 0.05$) compared to young, cognitively intact subjects. Aged rats also exhibited a particular reduction in mushroom spines (basilar: 31.5%, $P < 0.001$; apical: 21.4%, $P < 0.05$), a spine type associated with

greater synaptic stability and activity. In contrast, the density of filopodia in the aged group remained unchanged. Collectively, these findings identify distinct and novel decrements to structures in the aged hippocampal formation that are critical for the maintenance of neural function. Furthermore, this study characterizes these synaptic changes as a function of age-related cognitive decline.

B. Introduction

Cognitive decline often accompanies normal aging. For example, spatial memory impairment is commonly associated with aging in rodent, non-human primate, and human studies (Barnes, 1979; Gage et al., 1988; Gallagher et al., 1993; Herndon et al., 1997; Rapp et al., 1997; Lacreuse et al., 2005; Perlmutter et al., 1981; Sharps and Gollin, 1987; Uttl and Graf, 1993). While the role of the hippocampus in spatial memory formation has been studied extensively (Mahut and Zola, 1973; de Castro, 1974; Winson, 1978; Becker et al., 1980; Thompson, 1981; O'Keefe and Speakman, 1987), the role of the entorhinal cortex has only recently garnered similar attention. Lesion studies have demonstrated the necessity of the dorsal part of the medial entorhinal cortex for normal spatial memory acquisition and retention (Steffenach et al., 2005). Further studies have determined that layer II neurons in the dorsomedial entorhinal cortex produce complex place field activity (Fyhn et al., 2004;

Hafting et al., 2005; Sargolini et al., 2006). The integration of internal and external sensory input occurs in the medial entorhinal cortex, providing the source for place cell activity in the hippocampus (Sargolini et al., 2006; McNaughton et al., 2006). As the source of most hippocampal afferent projections, and the recipient of most hippocampal efferent projections, the entorhinal cortex regulates communication between the cortical mantle and the hippocampus (Hjorth-Simonsen and Jeune, 1972; Witter and Amaral, 1991; Witter et al., 2000). As a result, functional impairment of neurons in the entorhinal cortex could engender a more global cognitive decline.

Structural changes to the brain often underlie functional deterioration. Neuron loss in layer II of the entorhinal cortex is one of the earliest structural abnormalities to develop in Alzheimer's disease (Gomez-Isla et al., 1996). The early effect of the disease on these neurons may indicate their particular vulnerability to cellular insults. Normal aging, unlike Alzheimer's disease, is not associated with a decline in neuron number in the medial entorhinal cortex (Gazzaley et al., 1997; Merrill et al., 2000; 2001). Less dramatic structural changes have been linked to aging in other brain regions (Burke and Barnes, 2006). In particular, decreased postsynaptic spine density has been discovered in several areas, including the subiculum, anterior cingulate cortex, and prefrontal cortex (Uemura, 1985; Markham and Juraska, 2002; Wallace et al., 2007). Postsynaptic spines are small, motile structures that may be responsible for the formation and maintenance of memory at the cellular level. Both LTP-induction

and spatial learning increase the number of dendritic spines on hippocampal neurons (Moser et al., 1994; Trommald et al., 1996). Conversely, a loss of dendritic spines may impair LTP induction and memory formation.

Age-related changes to dendritic spines could occur in the form of either numeric losses or in perturbations of spine morphology. The structure of spines provides information regarding their activity and stability. Filopodia are the most motile spine type, and they may appear and retract within a single day (Dailey and Smith, 1996; Trachtenberg et al., 2002; Holtmaat et al., 2005). Filopodia are thought to provide the primary mechanism for new synapse formation (Ziv and Smith, 1996; Dailey and Smith, 1996; Fiala et al., 1998; Trachtenberg et al., 2002; Knott et al., 2006). Thin spines are more stable than filopodia, but also maintain significant plasticity (Matsuzaki et al., 2004; Popov et al., 2004; Noguchi et al., 2005). Spines with large spine heads, often called mushroom spines, are the most active and stable of the spine types (Trachtenberg et al., 2002; Grutzendler et al., 2002; Kasai et al., 2003; Holtmaat et al., 2005; Knott et al., 2006). These spines possess the largest post-synaptic densities (Harris and Stevens, 1988) with the greatest quantity of AMPA receptors (Nusser et al., 1998; Takumi et al., 1999; Matsuzaki et al., 2001). LTP-inducing stimuli increase spine head size and can convert thin spines to mushroom spines (Matsuzaki et al., 2004). This strengthening of neuronal associations may underlie memory formation. Conversely, decreased ability to form or maintain large

spines could lead to impaired memory in aged individuals. While decreased spine size has been linked to some neural disorders, including Down's syndrome and schizophrenia (Marin-Padilla et al., 1972; Roberts et al., 1996; Fiala et al., 2002), the effect of aging on spine morphology has not been investigated.

The present study tested the hypothesis that decrements in spine density and shape are associated with normal aging and age-related spatial memory impairment. We associated age-related cognitive impairment in spatial memory performance with a detailed anatomical study of identified layer II perforant path-projecting neurons of the dorsomedial entorhinal cortex. We now report extensive and significant reductions in spine density and shape in hippocampal formation neurons of cognitively impaired aged rats.

C. Materials and Methods

Behavioral testing

Twelve young (4 months old) and 18 aged (23 months old) adult male Fischer 344 rats were obtained from the Harlan/NIA rodent colony. Animal care procedures strictly adhered to the National Institutes of Health *Guidelines for the Care and Use of Experimental Animals* and were approved by the Institutional Animal Care and Use

Committee at the San Diego VA Medical Center. Morris water maze testing followed standard protocols (Morris, 1984; Merrill et al., 2001). Briefly, rats were tested for their ability to learn the location of a submerged, invisible platform using four high-contrast visual cues hung around the perimeter of a circular pool 6 feet in diameter. A white curtain surrounding the pool blocked additional visual input. The subjects' movements were collected by an automated tracking system (Videomex-One, Columbus Instruments, Columbus, OH). The acquisition (learning) and retention (memory) phases each consisted of 4 training blocks with 5 trials (maximum duration = 90 seconds per trial) and 1 probe trial (30 seconds per trial) in each block. For all hidden-platform trials, the platform was maintained 2 cm below the surface of the water in a static position. Before each probe trial, the platform was removed to enable evaluation of the rats' search strategies. Training in each phase took place over 8 days (3 trials per day) with a 17-day interval between the acquisition and retention phases. A cued-platform test, using a visible high-contrast platform in a new location, was given after completion of the hidden-platform testing. Animals with poor performance on the cued-platform test (visible platform) were excluded from the study. Water maze performance was analyzed using two measures. For hidden-platform trials, swim distance was averaged for each training block. For probe trials, the annulus crossings measure (the number of times the subject crossed the hidden platform location) was averaged for each phase. Statistical analysis of swim distance was performed using

repeated measures ANOVA. If the difference between groups was significant, individual testing blocks were further analyzed with the 2-tailed *t* test for unpaired samples. All data are presented as mean ± standard error of the mean (SEM).

Surgery

For spine density analysis, 7 young (4-6 months of age) and 12 aged (21-24 months of age) adult male Fischer 344 rats received injections of a retrograde label into the rostral dentate gyrus. This injection labeled neurons in layer II of the dorsomedial entorhinal cortex that compose the medial perforant path. The animals were anesthetized with 2.5% isoflurane (Baxter, Deerfield, IL) in oxygen at a flow rate of 0.8 L/min through a stereotaxic nose cone. The isoflurane concentration was varied based on individual animal response. Each rat received 4 injections (150 nL per injection) of red fluorescent microbeads (Retrobeads, Lumafluor Inc., Naples, FL) at a rate of 150 nL/min using a stereotaxically mounted Hamilton syringe (25s, 1.0 μL, Hamilton Co., Reno, NV). These injections were made into the septal pole (dorsal region) of the hippocampus using coordinates generated from a rat brain stereotaxic atlas (Paxinos and Watson, 1998). Bilateral injection coordinates were measured from Bregma and the surface of the brain (Site 1: anterior/posterior = -2.8 mm,

medial/lateral = ±1.1 mm, and dorsal/ventral = -4.2 mm; Site 2: anterior/posterior = -3.3 mm, medial/lateral = ±1.7 mm, and dorsal/ventral = -3.9 mm). The stereotax bite bar was adjusted to -4.3 mm for the flattest skull position allowed by the anesthesia nose cone.

Tissue Processing and Cell filling

Three days after injection of the retrograde label, the rats were anesthetized with a combination (2.5 mL/kg) of ketamine (25 mg/mL), xylazine (1.3 mg/mL), and acepromazine (0.25 mg/mL). Transcardial perfusion, optimized for cell filling, began with 9.25% sucrose solution (32°C) at 300 psi for 45 seconds, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, 32°C) at 300 psi for 47 seconds. The pressure was then reduced to 50 psi for 5 minutes. The brain was removed and post-fixed in ice-cold 4% paraformaldehyde for 1 hour.

Each hemisphere was individually blocked perpendicular to the medial entorhinal cortex pial surface. This was accomplished by cutting the hemisphere at a 20° angle from the horizontal (0° = rostral rhinal sulcus), with the plane of sectioning more dorsal at the rostral end and more ventral at the caudal end. This angle enabled sections of the dorsomedial entorhinal cortex to include the entire dendritic extent of

layer II neurons. The blocked tissue was cut to 200 μm-thick sections and collected in ice-cold 0.1 M PB for cell filling.

Cell filling was performed within 10 hours of perfusion. Sections were placed on gelatin-subbed slides, stabilized with squares of filter paper covering all but the medial entorhinal cortex, and immersed in Tris-buffered saline. Layer II of the dorsomedial entorhinal cortex was visible as a darkened band when projected to a video screen from an Olympus BX51WI light microscope (Olympus America Inc., Melville, NY) using infrared-differential interference contrast observation (IR-DIC) and a 5x Olympus MPlan objective. An Olympus LUMPlanFl/FR 40x objective was then used to identify neurons for cell filling based on the presence of red fluorescent microbeads clustered around the cell's nuclear envelope. Retrogradely labeled neurons were impaled with a sharp micropipette and filled by iontophoresis of 5% aqueous solution of lucifer yellow (LY; Sigma-Aldrich Co., St. Louis, MO). A current of 0.5-5 nA was used for 1-5 min. until the distal branches were filled and the spines were clearly visible. One to two neurons were filled in each section to ensure non-overlapping dendritic arbors. The sections were post-fixed in ice-cold 4% paraformaldehyde for 18 hours, then mounted and coverslipped with Fluoromount G (Southern Biotech, Birmingham, AL).

Dendritic branch imaging

Neurons selected for imaging and analysis had modified spiny stellate morphology typical of many excitatory layer II neurons in this region (Fig. 1A; Mikkonen et al., 2000) Layer II modified spiny stellate cells are characterized by a spherical or teardrop-shaped cell body; dendrites of equivalent size projecting radially from the cell body; and a single, larger dendrite projecting toward the pial surface, either directly or at an acute angle. The main "apical" branch was excluded from imaging and analysis, and the branches projecting directly from the main stalk were classified as first-degree apical branches. Apical dendrites and the smaller, radially projecting "basilar" dendrites were analyzed separately. Dendrite branch segments were collected as z-stack images using a multi-photon microscope with a Ti:sapphire laser tuned to 900 nm (FV300, Olympus America Inc., Melville, NY; Mai Tai IR laser, Newport Co., Mountain View, CA) (Fig. 1B). Sub-micron resolution of dendrites and dendritic spines was obtained with an Olympus LUMFL 60X 1.1 NA objective, 5-7x digital zoom, and 0.1 μm step size. The FluoView median filter was used on each image stack prior to analysis (Olympus America Inc., Melville, NY).

Analysis of spine density

A total of 11 neurons from the young group and 17 neurons from the aged group were used for basilar branch spine density analysis, and 8 neurons from young rats and 12 neurons from aged rats were used for apical spine density analysis. Dendrites selected for imaging and analysis consisted of second-degree through sixth-degree basilar and first-degree through fourth-degree apical branches longer than 10 μm. An average of 7 basilar and 2 apical branches were sampled from each neuron. The sampling of apical branches was limited by the small apical arbors in layer II neurons. Spine density was calculated as the number of spines per micron length of dendrite using maximal intensity two-dimensional (2D) projections from 3D z-stack images. Dendrite lengths were measured using the NeuronJ plug-in (version 1.1.0; Erik Meijering) for NIH ImageJ software (version ImageJ 1.37v; Wayne Rasband, NIH, Bethesda, MD). Spine density was calculated for each branch, and the average spine density of each neuron was determined. The differences in average spine density between groups were evaluated with the 2-tailed *t* test for unpaired samples.

Spine classification and analysis of spine morphology

Spines were divided into four categories based on morphological features, as previously described (Harris et al., 1992; De Simoni et al., 2003; Tyler and Pozzo-Miller, 2003). Figure 1C provides a schematic representation of the categories: stubby (type I), mushroom (type II), thin (type III), and filopodia (type IV). Stubby spines had no apparent neck and were defined as short protrusions with a length that was no greater than their width. Spines were classified as mushroom, spines if they had a large head with a diameter more than twice their neck diameter. Thin spines were also characterized by a clearly defined head and neck, but the ratio of head to neck diameter was less than 2. For the cases in which the neck was indistinct, the maximum head diameter for thin spines was set at 0.675 μm (spherical volume: 0.16 μm^3) based on the measured head diameters of 50 fully visible thin and mushroom spines. This measure is similar to that used by Komatsuzaki et al., 2005 (0.6-μm maximum head diameter) and the 0.1-μm^3 maximum spine head volume selected by Noguchi et al., 2005. Type IV spines, or filopodia, were identified as longer protrusions having a greater length than width and lacking a clearly defined head.

The density of each spine type was determined using the methods and branches described for the analysis of overall spine density. Spine-type densities were calculated for each branch. Additionally, the percentage of each spine type relative to

the total spine count was determined for each branch. Group differences were analyzed using the 2-tailed *t* test for unpaired samples. All water maze and spine density analyses were performed blinded.

D. Results

Aged male Fischer 344 rats consistently exhibit spatial memory impairment

Spatial memory impairment is common in aged rats, but the prevalence of this cognitive decline has not been thoroughly investigated in the Fischer 344 rat strain. To evaluate the water maze performances of young (4 mos.) and aged (23 mos.) male Fischer rats in the hidden-platform trials, we compared mean swim distances in 8 testing blocks. The mean swim distance for aged rats was significantly greater than for young rats in the acquisition (repeated measures ANOVA: $F_{1,28} = 57.69$, $P < 0.001$; Fig. 2A) and retention phases ($F_{1,28} = 32.46$, $P < 0.001$; Fig. 2B). Analysis of individual blocks revealed that the mean swim distance for aged rats was significantly greater than for young rats starting with block 2 of the acquisition phase (unpaired 2-tailed *t* test: $t_{28} = -5.24$, $P < 0.001$; Fig. 2A) and continuing through all additional testing blocks (block 3: $t_{28} = -6.00$, $P < 0.001$; block 4: $t_{28} = -5.25$, $P < 0.001$; block 5: $t_{28} = -5.02$, $P < 0.001$; block 6: $t_{28} = -4.29$, $P < 0.001$; block 7: $t_{28} = -3.96$, $P < 0.001$;

block 8: $t_{28} = -5.22$, $P < 0.001$; Fig. 2A and 2B). These data indicated ongoing spatial memory impairment in the aged rats as a group.

We then assessed the aged rats individually to determine the proportion of aged subjects that develop spatial memory impairment by 23 months. Aged rats were categorized as aged-unimpaired if their individual mean swim distances in blocks 4 and 5 were within 2 standard deviations of the group mean swim distances for young rats in the same blocks. Only 2 of the 18 aged rats (11.1%) met these criteria. The remaining rats were labeled as aged-impaired. Figure 2 (C and D) demonstrates the results of hidden platform training following separation of the aged rats into unimpaired and impaired groups. Because the aged-unimpaired group contained only 2 subjects, we limited our statistical analysis to the young and aged-impaired groups. Group performances differed significantly for both acquisition (repeated measures ANOVA: $F_{1,26} = 61.72$, $P < 0.001$) and retention phases ($F_{1,26} = 40.18$, $P < 0.001$). Comparisons between the young and aged-impaired groups showed significant differences in block 2 (unpaired 2-tailed *t* test: $t_{26} = -5.14$, $P < 0.001$) through block 8 (block 3: $t_{26} = -6.04$, $P < 0.001$; block 4: $t_{26} = -6.21$, $P < 0.001$; block 5: $t_{26} = -5.88$, $P < 0.001$; block 6: $t_{26} = -4.28$, $P < 0.001$; block 7: $t_{26} = -4.24$, $P < 0.001$; block 8: $t_{26} = -5.85$, $P < 0.001$). These data demonstrate that marked spatial memory decline occurs in at least 89% of 23-month-old male Fischer rats.

The unimpaired group appeared to perform better than the impaired group on block 4 of acquisition testing and throughout most of retention testing. To clarify whether the swim distance measure for the aged-unimpaired group indicates successful spatial learning or the development of an alternative effective search strategy, we examined annulus crossings from the probe trials. This measure describes the number of times the rat crosses over the standard hidden platform position after the platform has been removed. Perseveration in the platform position, demonstrated by a high number of annulus crossings, indicates that the subject has learned to use the visual cues to identify the correct platform location. The mean annulus crossings measure was low for both aged-impaired and aged-unimpaired groups during acquisition and retention testing (Fig. 2E and 2F). When paired with the results from the hidden-platform testing, these data indicate that the aged-unimpaired rats have learned an effective search strategy for the Morris water maze, but are not employing spatial memory cues as effectively as young subjects in learning this task.

Excitatory layer II neurons of the medial perforant path undergo an age-related reduction in spine density

Next, we investigated structural changes to layer II neurons of the dorsomedial entorhinal cortex that are known to play an important role in spatial memory

(Steffanach et al., 2004; Fyhn et al., 2004). Neuron number is stable in the rat and monkey entorhinal cortex with aging (Gazzaley et al., 1997; Merrill et al., 2000; 2001), but structural perturbation of neurons may underlie the behavioral decline. To investigate this possibility, we measured the density of dendritic spines on modified spiny stellate neurons that project to the rostral dentate gyrus.

Quantitative analysis revealed that the mean spine density in aged rats was significantly lower than in young rats on both basilar (unpaired 2-tailed t test: $t_{26} = 4.81$, $P < 0.001$) and apical dendrites ($t_{18} = 2.24$, $P = 0.04$). Figure 3 provides representative branches from each group. Basilar branches exhibited an 18.2% age-related decrease in spine density, and the apical branches showed a similar 16.0% decrease.

Aging is associated with a specific loss of mushroom spines

The morphology of a postsynaptic spine provides information regarding the size of its postsynaptic density (Harris and Stevens, 1988), its stability (Trachtenberg et al., 2002; Kasai et al., 2003), and the activity of its synapse (Matsuzaki et al., 2001; Matsuzaki et al., 2004; Sapoznik et al., 2006). To investigate the effect of aging on individual spine types, we first determined the density of each type. On the basilar branches, the stubby (type I), mushroom (type II), and thin (type III) spines decreased

in density in the aged rats, with the type-II, mushroom spines showing the greatest decrease of 31.5% (Table 1). The density of filopodia (type IV) in aged rats did not differ from that of the young rats ($t_{186} = 1.35$, $P = 0.18$). Aging had similar effects on apical spine types, however only the density of mushroom spines decreased significantly in the aged rats (Table 1).

To further clarify whether aging causes a change in the prevalence of different spine morphologies, we calculated, for each branch, the percent of the spine population represented by each spine type. Of the four spine types, only mushroom spines differed between the young and aged animals. The aged rats had a lower percentage of mushroom spines than the young rats on basilar branches (Fig. 4). Aging did not significantly alter the spine type percentages on apical branches.

E. Discussion

The present study indicates that aging is associated with a significant loss of both basilar and apical spines in layer-II hippocampus-projecting neurons of the entorhinal cortex, and a particular reduction of mushroom spines. Mushroom spines are the most active, and typically the most stable (Trachtenberg et al., 2002), spines with the largest spine heads. Although reduced spine size has been identified in some neural disorders, including schizophrenia and Down's syndrome (Roberts et al., 1996;

Marin-Padilla et al., 1972), the results described in this study provide the first evidence that a reduction in spine size accompanies normal aging.

The specific decline in mushroom (type II) spines with aging suggests two neural mechanisms that could be subjects for further study in the context of understanding age-related cognitive decline. First, the ability of a neuron to produce and maintain large postsynaptic densities may decrease with age. While the ratio of mushroom spines to the total spine population decreased on basilar branches, there was also a non-significant increase in the representation of stubby (type I) and thin (type III) spines (Fig. 4). The maintenance of thin spines with smaller spine heads and postsynaptic densities would hypothetically require less energy and resource expenditure than mushroom spines. Findings of reduced mitochondrial function in aged neurons (Melov, 2004) and decreased protein synthesis in the entorhinal cortex with aging (Kelly et al., 2000) support this possibility. Second, the stability and activity of presynaptic inputs to the layer II neurons in the medial entorhinal cortex may decrease with age. Many studies have demonstrated that the size of a spine head is related to synaptic activity (Fifkova and VanHarreveld, 1977; Matsuzaki et al., 2001; Matsuzaki et al., 2004; Sapoznik et al., 2006; Zhou et al., 2004). While LTP-inducing stimuli increase spine size (Fifkova and VanHarreveld, 1977; Matsuzaki et al., 2004), the induction of long-term depression causes spines to shrink (Zhou et al., 2004). Decreased presynaptic activity from other brain regions could result in reduced

spine head size on aged medial entorhinal cortex neurons and might alter the relative proportions of mushroom and thin spines.

Fewer or less active synaptic inputs to layer II medial entorhinal cortex neurons in aged rats may underlie the decreased densities of stubby, mushroom, and thin spines on the basilar dendrites. Although mushroom spines display the largest age-related change, the densities of stubby and thin spines also decrease (Table 1). Apical branches show a similar, but non-significant decrease in these measures. Recent evidence suggests that spines form new synapses with pre-existing active boutons (Toni et al., 1999; Toni et al., 2007) Weakened activity in a presynaptic terminal may decrease the number of synapses that can be established and maintained. Similarly, a decrease in the number of presynaptic inputs to aged perforant path neurons could also contribute to an overall decline in spine density. The finding of decreased synaptophysin and synapsin synthesis in the aged rat entorhinal cortex supports this possibility (Kelly et al., 2000).

The literature suggests a third potential source for age-related spine density decline; aged neurons may be impaired in their ability to generate new spines. If so, the slow turnover of stable spines that occurs normally (Trachtenberg et al., 2002; Grutzendler et al., 2002; Holtmaat et al., 2005) would eventually lead to a deficit. The clearest indication of this mechanism in our fixed-tissue study would be a decrease in filopodia (type IV spines). These spines are the most plastic of spine types, extending

and retracting within a single day (Dailey and Smith, 1996; Trachtenberg et al., 2002), and are believed to initiate synapse formation with existing presynaptic inputs (Ziv et al., 1996; Fiala et al., 1998; Knott et al., 2006). This study does not indicate a decrease in type IV spine density on either the apical or basilar branches of medial entorhinal cortex layer II neurons. Because of the volatility of filopodia, fixed-tissue techniques may be insensitive to relevant age-related changes in the plasticity of this spine type. This mechanism would be more clearly addressed in a live preparation.

Whether the spine density declines on basilar and apical branches reflect changes in the same or different afferent synaptic connections remains unclear. A number of regions project to these neurons, including the olfactory area, superior temporal gyrus, parasubiculum, perirhinal cortex, parahippocampal cortex, and presubiculum (Insausti et al., 1987; Witter, 1993; Mikkonen, 1999). Layer-II neurons of the medial entorhinal cortex also receive direct input from occipital and parietal cortices (Kerr et al., 2007), and collateral projections from neighboring layer II excitatory neurons (Kohler, 1986; Lingenhohl and Finch, 1991; Buckmaster et al., 2004) that form recurrent excitatory synapses (Kumar et al., 2007). To date, studies of afferent projections to the medial entorhinal cortex are limited in resolution to the layer in which the neurons synapse. In this study, most of the apical and basilar dendrites collected for analysis resided within layer II. Further study will be necessary

to resolve whether the presynaptic excitatory inputs to modified spiny stellate neurons in the entorhinal cortex differ between apical and basilar dendrites.

The medial entorhinal cortex is an important cortical region that serves as the primary interface between the neocortex and the hippocampus. As a result of this unique functional role, changes to entorhinal function could have widespread cognitive consequences. Water maze testing in the present study demonstrated that aging disrupts the activity of this region. This study also established that aging leads to a decrease in the quantity and stability of excitatory connections with layer-II medial entorhinal cortical neurons with a level of individual cell resolution and detail not previously reported. Given its important functional role in memory, widespread projections to hippocampal and extra-hippocampal structures, and distinct structural and functional changes with aging, the entorhinal cortex may provide an optimal target for testing therapeutic interventions in age-related cognitive decline.

F. Figures

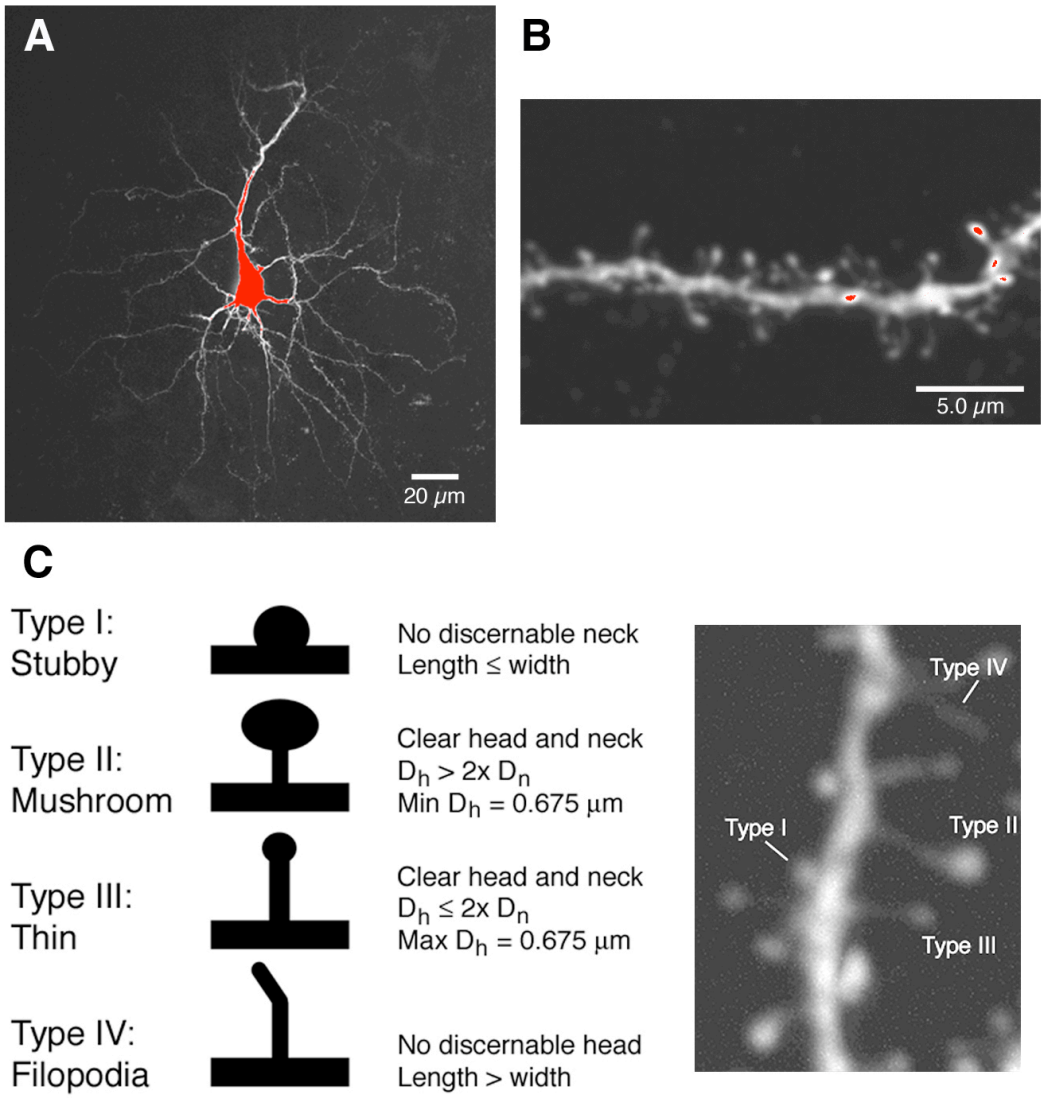

Figure 1. Representative images of a lucifer yellow (LY)-filled neuron and dendritic branch used for spine density and spine type analyses. **(A)** A typical LY-filled neuron in layer II of the dorsomedial entorhinal cortex. **(B)** LY-filled basilar dendrite segment. **(C)** Criteria for spine type classification including a schematic representation and real example of each spine type.

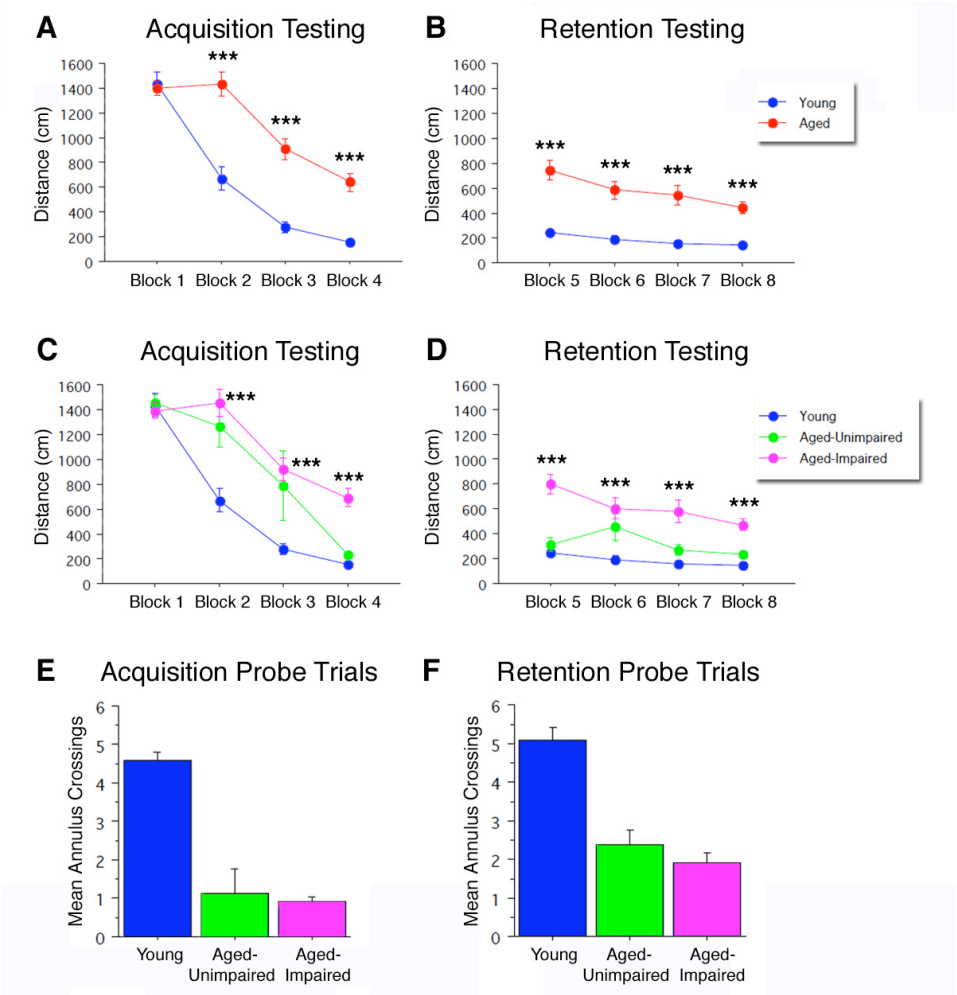

Figure 2. Spatial memory performance of young and aged male Fischer 344 rats. Acquisition **(A)** and retention **(B)** testing in the Morris water maze consisted of 4 training blocks separated by a 17-day interval. Performance was based on mean swim distance measures. Asterisks indicate a significant difference relative to the young group (repeated measures ANOVA with post hoc unpaired 2-tailed *t* test; ***$P <$ 0.001). Error bars represent SEM. **(C,D)** Aged rats were separated into aged-unimpaired and aged-impaired groups based on individual performance in blocks 4 and 5. Statistical analysis was performed only on young and aged-impaired groups. Asterisks indicate a significant difference relative to the young group (repeated measures ANOVA with post hoc unpaired 2-tailed *t* test; ***$P < 0.001$). **(E,F)** Search strategy was evaluated using mean annulus crossings measure in water maze acquisition **(E)** and retention **(F)** probe trials.

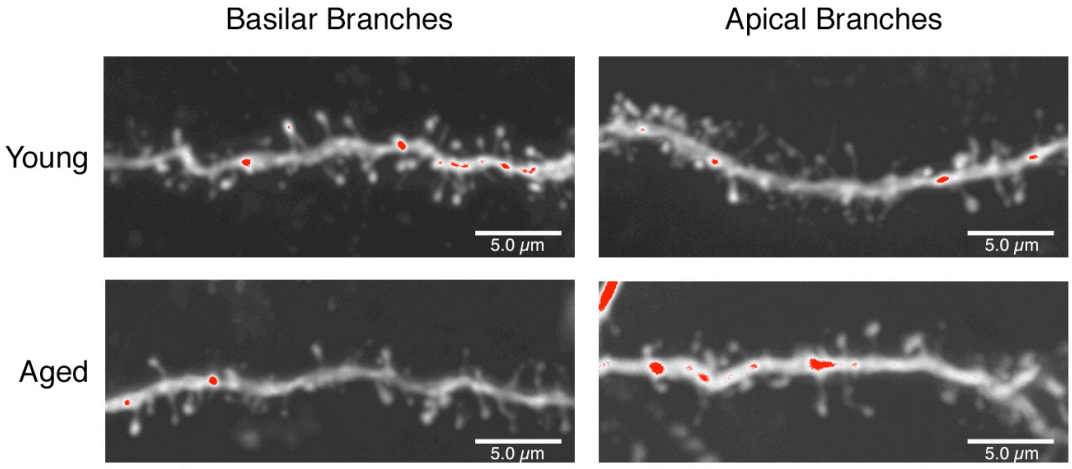

Figure 3. Representative images of lucifer yellow-filled basilar and apical dendrite branches from young and aged rats.

Table 1. Spine density measurements of layer II modified spiny stellate neurons

Parameter	Young	Aged	% Difference
Basilar branches			
All (neuron avg)	1.76 ± 0.06	1.44 ± 0.04**	-18.2
By Type (branch avg)			
Mushroom	0.54 ± 0.02	0.37 ± 0.01**	-31.5
Stubby	0.46 ± 0.02	0.41 ± 0.02**	-10.9
Thin	0.73 ± 0.02	0.61 ± 0.02**	-16.4
Filopodia	0.07 ± 0.01	0.06 ± 0.01	-14.3
Apical branches			
All (neuron avg)	1.94 ± 0.11	1.63 ± 0.09**	-16.0
By Type (branch avg)			
Mushroom	0.56 ± 0.05	0.44 ± 0.04**	-21.4
Stubby	0.52 ± 0.04	0.44 ± 0.02	-15.4
Thin	0.86 ± 0.07	0.76 ± 0.05	-11.6
Filopodia	0.07 ± 0.01	0.07 ± 0.01	0

Spine density measurements are listed as mean spine density (spines per μm) ± SEM. Asterisks indicate differences in spine density relative to the young group (unpaired 2-tailed *t* test; $^{**}P < 0.05$). The % Difference column indicates the percent change in spine density of the aged group relative to young. Both basilar and apical branches exhibited a significant age-related decrease in spine density, whereas the effect of aging on individual spine types varied.

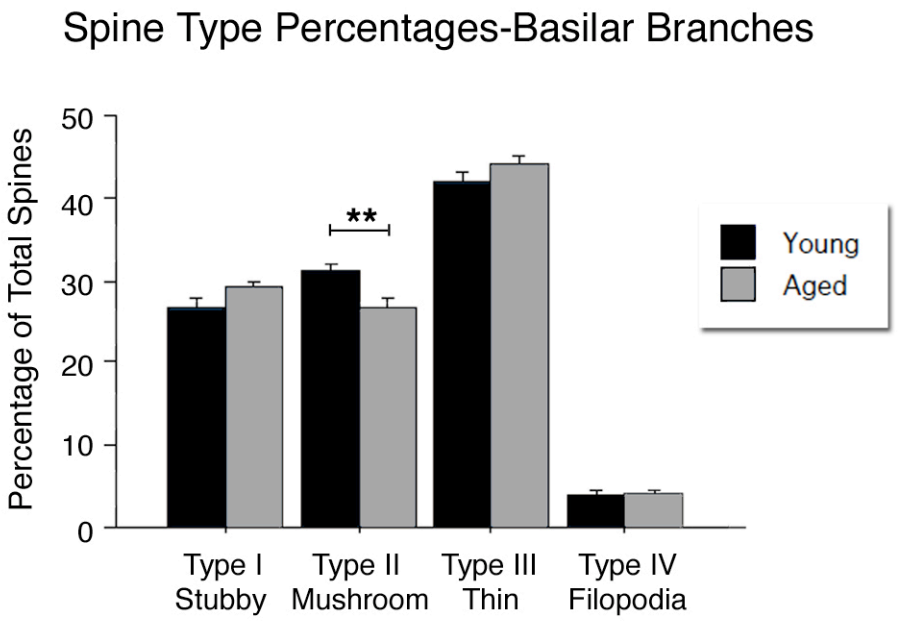

Figure 4. Percent of spine population represented by each spine type on basilar branches. Although the spine density of stubby, mushroom, and thin spines on basilar branches decreased in aged rats, only mushroom spines represented a smaller proportion of total spines in the aged group relative to the young.

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H. Chapter Acknowledgements

Chapter 2, in full, is in preparation for publication. Tonya Mead and Mark Tuszynski are the authors of this manuscript. The dissertation author was the primary investigator and first author of this paper.

III

BDNF Reverses Age-Related Degeneration of Synaptic Structures in the Hippocampal Formation

A. Abstract

Normal aging is associated with changes in neuronal structure that impair synaptic function. We previously reported that perforant path-projecting neurons in the dorsomedial entorhinal cortex of aged rats exhibit significant reductions in basilar and apical spine density, and losses of mushroom-shaped spines that contain the largest and most active synapses. Brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) influence adult cortical neuron plasticity by modifying synaptic activity and neuronal structure. Although cortical levels of BDNF and NGF do not decline with normal aging, we hypothesized that over-expression of BDNF or NGF in neurons of the aged rat medial entorhinal cortex could, via trophic mechanisms, normalize age-related declines in spine density. We now report that lentiviral-mediated delivery of BDNF to the aged rat entorhinal cortex entirely reverses age-related declines in spine density and restores the relative proportions of spine types to

those of young subjects. In contrast, NGF gene delivery has no effect on these parameters. Further, BDNF gene delivery to the entorhinal cortex enhanced hippocampal levels of the synaptic protein synaptophysin. These findings highlight the ability of BDNF to modify mechanisms associated with synaptic degeneration in the aged brain.

B. Introduction

Brain-derived neurotrophic factor (BDNF) has a profound influence on neuronal plasticity in the adult brain. Presynaptically, BDNF rapidly facilitates neurotransmitter release by increasing intracellular calcium concentration (Stoop and Poo, 1996; Li et al., 1998) and by increasing vesicle docking at active zones of excitatory synapses (Tartaglia et al., 2001). On postsynaptic terminals, BDNF enhances glutamate-evoked responses by increasing NMDA-channel open probability (Levine et al., 1998). Depending on experimental conditions, BDNF either augments tetanus-induced hippocampal and cortical long-term potentiation (LTP) (Figurov et al., 1996; Akaneya et al., 1997), or independently induces LTP (Akaneya et al., 1997; Messaoudi et al., 1998; Messaoudi et al., 2002).

In addition to enhancing synaptic transmission, BDNF can also influence the structural plasticity of neurons. In previous in vitro studies, BDNF increased spine

turnover (Horch et al., 1999) and increased the spine density of Purkinje and hippocampal pyramidal neurons (Shimada et al., 1998; Tyler and Pozzo-Miller, 2001; Tyler and Pozzo-Miller, 2003). The increase in hippocampal spine density was dependent on the MAPK/ERK signal transduction pathway (Alonso et al., 2004) and was modulated by cAMP (Ji et al., 2005). Spine morphology was also responsive to BDNF (Tyler and Pozzo-Miller, 2003; Matsutani and Yamamoto, 2004). Because of its ability to modify adult neuron plasticity in vitro, we hypothesized that BDNF can reverse the structural deterioration of aged neurons in vivo.

BDNF and NGF are members of the same family of neurotrophic proteins, but they bind preferentially to different receptors, TrkB and TrkA, respectively. Whereas the BDNF receptor, TrkB, is expressed by glutamatergic neurons in adult cortex (Fryer et al., 1996; Yan et al., 1997; Numakawa et al., 1999), TrkA is found on cholinergic terminals projecting from the basal forebrain to neocortical neurons (Holtzman et al., 1992; Steininger et al., 1993; Sobreviela et al., 1994; Holtzman et al., 1995; Prakash et al., 2004). Because of the difference in receptor location, BDNF and NGF have different mechanisms of action. Whereas BDNF has a direct influence on cortical neurons, NGF indirectly influences these neurons by increasing the activation of their cholinergic inputs (Prakash et al., 2004; Prakash et al., 2005). In previous studies, intracerebroventricular infusions of NGF protein into adult (Kolb et al. 1997) and aged rats (Mervis et al., 1991) increased the terminal-branch spine density of

cortical pyramidal neurons. In the present study, we tested whether more restricted administration of NGF through intraparenchymal lentiviral vector injections would have a similar effect on aged rat spine density.

Age-related cognitive decline, specifically spatial memory impairment, is associated with neuronal deterioration in the hippocampal formation (Geinisman et al., 1986; Nicholson et al., 2004; Mead and Tuszynski, 2008). In a previous study, we determined that with normal aging, perforant path-projecting neurons of the dorsomedial entorhinal cortex exhibit decreases in spine density of 18.2% on their basilar branches and 16.0% on their apical branches (Mead and Tuszynski, 2008). Mushroom spines, the largest and most active spines, experienced the greatest age-related decrease. These well-characterized structural changes provided an ideal background for testing the therapeutic potential of neurotrophins in a model of normal aging. In the present study, we administered lentiviral vectors expressing BDNF or NGF to the dorsomedial entorhinal cortex in aged rats to examine the effects of enhanced neurotrophin expression on aged neuron spine density and morphology.

Additionally, age-related decreases in synapse number in the middle molecular layer of the dentate gyrus (Geinisman et al., 1992), a termination zone for entorhinal projections, provided us with a substrate for testing whether BDNF can orchestrate synaptic changes through anterograde transport. Furthermore, age-related decline in and synaptophysin expression in the same region (Davies et al., 2003) enabled us to

test whether BDNF can reverse age-related changes in synaptic protein expression. To test these possibilities, we measured the density of synaptophysin-expressing terminals and the intensity of synaptophysin expression in the aged dentate gyrus with and without BDNF administration in the entorhinal cortex.

Combined, these studies provide insight into the influence of BDNF on synaptic structures in the aged hippocampal formation.

C. Results

A total of 7 young (4-6 months old) and 31 aged (21-24 months old) male Fischer 344 rats were used in the present study. For cell filling, 31 aged rats were divided into four groups. Three groups received intraparenchymal injections of lentiviral vectors expressing BDNF (n = 7), NGF (n = 6), or the reporter gene LacZ (n = 6) into the dorsomedial entorhinal cortex. A fourth group of 12 naïve, aged rats served as unoperated controls. Comparisons were made to 7 unoperated young rats. For spine density analysis, the basilar branches of 71 neurons (young = 11, aged = 17, aged-BDNF = 19, aged-NGF = 12, aged-LacZ = 12) and the apical branches of 55 neurons (young = 8, aged = 12, aged-BDNF = 14, aged-NGF = 9, aged-LacZ = 12) were measured. Spines were classified by morphology on a total of 436 basilar branches (young = 89, aged = 99, aged-BDNF = 123, aged-NGF = 72, aged-LacZ =

53) and 151 apical branches (young = 23, aged = 24, aged-BDNF = 39, aged-NGF = 27, aged-LacZ = 38).

Increased BDNF Expression Reverses Aged Spine Density Decline

In a previous study, we determined that there was a significant age-related decrement in spine density in layer II neurons of the dorsomedial entorhinal cortex (Mead and Tuszynski, 2008). To test the response of aged neurons to increased neurotrophin expression, lentiviral vectors (100 μg/mL p24) expressing BDNF, NGF, or LacZ were administered bilaterally to this region in 19 aged rats. Vector injection resulted in expression of the inserted gene throughout the dorsal portion of the medial entorhinal cortex (Fig. 1). All young and aged rats received injections of red fluorescent microbeads into the rostral hippocampus to retrogradely label neurons projecting from the dorsomedial entorhinal cortex; these neurons were then filled in entorhinal-hippocampal slices with lucifer yellow (LY). LY-filled neurons had modified spiny stellate morphology with a round or teardrop-shaped cell body, radially projecting dendrites of equivalent initial diameter, and a single, thicker dendrite projecting toward the pial surface (Fig. 2A; Mikkonen et al., 2000). Three-dimensional images of the apical and basilar branches were collected using multi-

photon microscopy, and spine density was measured from two-dimensional maximum-intensity projections of the three-dimensional images (Fig. 2B and 3B).

BDNF expression resulted in a large and significant increase in the density of dendritic spines in aged rats. While all three aged groups that underwent lentiviral vector injections exhibited at least a slight elevation of spine density compared to non-injected, aged controls, only BDNF lentiviral vector expression entirely reversed age-related decrements in spine density on both basilar (Fig. 3A) and apical (Fig. 3C) branches (basilar branches, 1-way ANOVA: $F_{4,66} = 12.06$, $P < 0.001$, post-hoc Fischer's aged-BDNF vs. aged: $P < 0.001$; apical branches, 1-way ANOVA: $F_{4,50} = 8.22$, $P < 0.001$, post-hoc Fischer's aged-BDNF vs. aged: $P < 0.001$). The spine density of aged-BDNF rats was also significantly greater than that of the aged-LacZ control group ($P < 0.001$).

The effect of the NGF lentiviral vector in aged rats did not differ from the control LacZ vector, and aged-NGF rats had significantly lower spine densities than aged-BDNF rats (Fig. 3A and C; $P \leq 0.001$). However, the basilar branch spine density measurements from both aged-NGF and aged-LacZ rats were comparable to the young group (Fig. 3A; $P > 0.05$), and apical branch spine density in these groups did not differ significantly from either young or aged rats (Fig. 3C; $P > 0.05$). The similarities between the aged-NGF and aged-LacZ spine density measurements

suggest that NGF expression is not responsible for the nominal increase in aged rat spine density observed in the aged-NGF group.

BDNF, not NGF, Increases the Density of Mushroom and Thin Spines on Aged Neurons

Previously, we demonstrated that aging leads to a significant decrease in stubby (type I), mushroom (type II), and thin (type III) spines on the basilar branches of layer II dorsomedial entorhinal cortex neurons (Mead and Tuszynski, 2008; see Fig. 2C for spine type criteria). Apical branches displayed similar changes, but the decrease was significant only for mushroom spines. In the current study, we have investigated the effects of BDNF, NGF, and LacZ lentiviral vector expression on the densities of these three individual spine types in aged neurons.

Comparisons between the aged-BDNF group and the naïve aged controls showed that BDNF lentiviral vector expression in aged rats increased the density of all three spine types on basilar dendrites (Fig. 4A-C; stubby spines, 1-way ANOVA: $F_{4,431} = 6.18$, $P < 0.001$, post-hoc Fischer's aged vs. aged-BDNF: $P < 0.001$; mushroom spines: $F_{4,431} = 31.37$, $P < 0.001$, post-hoc: $P < 0.001$; thin spines: $F_{4,431} = 14.37$, $P < 0.001$, post-hoc: $P < 0.001$). BDNF lentiviral vector administration also increased the density of mushroom and thin spine types on apical dendrites (Fig. 4E

and F; mushroom spines, 1-way ANOVA: $F_{4,146} = 6.75$, $P < 0.001$, post-hoc Fischer's aged vs. aged-BDNF: $P < 0.001$; thin spines: $F_{4,146} = 5.10$, $P < 0.001$, post-hoc: $P = 0.003$). The group differences in apical-branch stubby spine density, on the other hand, did not reach significance (Fig. 4D; 1-way ANOVA: $F_{4,146} = 2.03$, $P = 0.09$).

BDNF had a greater effect on mushroom and thin spines than either NGF or LacZ (Fig. 4B, C, E, and F). For stubby spines, on the other hand, aged-BDNF spine density did not differ from either aged-NGF or aged-LacZ spine densities on basilar branches (Fig. 4A; $P > 0.05$), and group changes in the density of stubby spines did not reach significance on apical branches (Fig. 4D; 1-way ANOVA: $F_{4,146} = 2.03$, $P = 0.09$). Although BDNF lentiviral vector administration stimulated an increase in the densities of all three spine types, these results suggest that only the changes to mushroom and thin spines were specific to BDNF expression.

Unlike BDNF expression, NGF expression did not increase the density of individual spine types on aged medial entorhinal cortex neurons. Mushroom spine density in the aged-NGF group did not differ from either the aged-LacZ rats or the naïve aged control group (Fig. 4B and E; $P > 0.05$). Thin spine density also did not differ between these groups on apical dendrites. (Fig. 4F). On basilar branches, thin spine density in the aged-NGF and aged-LacZ groups did not differ (Fig. 4C), but were significantly greater than in aged control rats (post-hoc Fischer's aged-NGF vs. aged: $P = 0.002$; aged-LacZ vs. aged: $P = 0.03$). The similarity in this measure

between aged-NGF and aged-LacZ rats suggests that lentiviral vector administration, independent of NGF expression, caused the increase in the aged-NGF group. An analogous change occurred in measures of stubby spines (Fig. 4A). Stubby spine density on basilar branches was comparable for all three aged virus-injected groups ($P > 0.05$); all three groups had density measurements that were comparable to that of the young group ($P > 0.05$); and all three groups had significantly greater spine densities than that of the aged group (post-hoc Fischer's aged vs. aged-BDNF: $P < 0.001$, aged vs. aged-NGF: $P = 0.01$, aged vs. aged-LacZ: $P = 0.002$). These data demonstrate that lentiviral vector injections caused small increases in the densities of stubby and thin spines on basilar branches, but NGF expression had no additional effect on spine density.

BDNF Restores Proportions of Spine Types to Patterns in Young Subjects

Spine density measurements identify changes in each spine type, but age and treatment may affect spine types differentially. Thus, for each group, we calculated the percent of the total spine population (on each branch) represented by each spine type. Aging resulted in a significant increase in the percentage of stubby spines (Fig. 5A; 1-way ANOVA: $F_{4,431} = 5.53$, $P < 0.001$, post-hoc Fischer's aged vs. young: $P = 0.04$) and a significant reduction in the percentage of mushroom spines (Fig. 5B; $F_{4,431} =$

8.66, $P < 0.001$, post-hoc: $P < 0.001$) on basilar dendrites. Lentiviral vector administration of BDNF, but not NGF, reversed these age-related changes (Fig. 5A and B). The percentage of thin spines on basilar branches was not affected by either aging or neurotrophin expression (1-way ANOVA: $F_{4,431} = 1.76$, $P = 0.14$; data not shown).

BDNF Restores Synaptophysin Levels in Hippocampus

Measurements of synaptophysin immunolabeling demonstrated a significant age-related decline in the density of synaptophysin-expressing presynaptic terminals in the middle (MML), inner (IML), and outer (OML) molecular layers of the dentate gyrus (Fig. 6A-C; MML, 1-way ANOVA: $F_{2,15} = 31.08$, $P < 0.001$, post-hoc Fischer's aged-EGFP vs. young: $P < 0.001$; IML: $F_{2,15} = 19.73$, $P < 0.001$, post-hoc: $P < 0.001$; OML: $F_{2,15} = 9.32$, $P = 0.002$, post-hoc: $P < 0.001$). The age-related decrease in synaptophysin density was not caused by a decrease in the size of synaptophysin-positive presynaptic terminals. In fact, aging was associated with an increase in mean terminal size (1-way ANOVA: $F_{2,15} = 4.486$, $P = 0.03$, post-hoc Fischer's aged-EGFP vs. young: $P = 0.01$). Synaptophysin intensity in the dentate molecular layer also

demonstrated a significant age-related decline (Fig. 6D; 1-way ANOVA: $F_{2,15} = 14.03$, $P < 0.001$, post-hoc Fischer's aged-EGFP vs. young: $P < 0.001$).

Lentiviral-mediated BDNF gene delivery to the dorsomedial entorhinal cortex significantly increased the density of synaptophysin-labeled synaptic terminals in the middle ($P < 0.001$) and inner ($P < 0.001$) molecular layers (Fig. 4A-C). The effect of BDNF on terminals in the outer molecular layer did not reach significance ($P = 0.11$). BDNF administration in the entorhinal cortex also increased the intensity of synaptophysin immunolabeling in the dentate molecular layer (Fig. 4D; $P < 0.001$).

D. Discussion

This study demonstrates that BDNF gene delivery to the entorhinal cortex can reverse synaptic changes in the hippocampal formation that occur with normal aging. Previously (Mead and Tuszynski, 2008), we determined that medial entorhinal cortical neurons exhibit age-related decrements in spine density. In this study, we demonstrated that administration of BDNF lentiviral vector to the entorhinal cortex increased spine density, reversing the age-related decline.

BDNF lentiviral vector administration in the aged entorhinal cortex also resulted in significant changes in the relative proportions of stubby and mushroom spines. Spine structure is modifiable, and correlates with the maturity, activity, and

stability of the synapse. The prevalence of stubby spines during early development suggests that this spine type is typically associated with an immature synapse (Fiala et al., 1998; Harris et al., 1992). Recent studies investigating the relationship between activity and spine type in the adult hippocampus support this supposition. When synaptic transmission was blocked in adult hippocampal slices, stubby spines and filopodia proliferated (Petrak et al., 2005). On the other hand, LTP induction of the perforant path in vivo decreased the proportion of synapses on stubby spines in the middle molecular layer of the dentate gyrus (Popov et al., 2004). Whereas stubby spines suggest the presence of immature synapses, mushroom spines indicate the presence of highly active and stable synapses (Trachtenberg et al., 2002; Kasai et al., 2003; Holtmaat et al., 2005; Knott et al., 2006). Mushroom spines possess the largest postsynaptic densities (Harris and Stevens, 1988) with the greatest quantity of AMPA receptors (Nusser et al., 1998; Takumi et al., 1999; Matsuzaki et al., 2001). In the present study, aged neurons in the entorhinal cortex experienced a significant reduction in the proportion of mushroom spines and an increase in the relative proportion of stubby spines suggesting a relative decline in mature and functional synapses. Increased BDNF expression entirely reversed these age-related changes.

We also present evidence that BDNF administration in the entorhinal cortex can reverse age-related synaptic changes in the dentate gyrus, a region to which entorhinal neurons project. The unique properties of BDNF enable it to address age-

related changes in presynaptic terminals, in addition to its effects on postsynaptic spines. Since BDNF undergoes anterograde transport (see Conner et al., 1998; Tonra, 1999 for review), BDNF lentiviral vector delivery to the medial entorhinal cortex could influence neurons within the hippocampal formation that receive afferent projections from the entorhinal cortex. Indeed, we determined that age-related decrements in synaptophysin expression in the dentate gyrus, a target of entorhinal projections, exhibited partial recovery following BDNF administration in the entorhinal cortex. Exogenous BDNF expression in the entorhinal cortex increased the density of synaptophysin-expressing presynaptic terminals in the middle and inner molecular layers of the dentate gyrus. In the current study, both layers exhibit significant age-related declines in the density of synaptophysin-labeled terminals, and, in a previous study, these regions showed a significant age-related decline in axospinous synapse number (Geinisman et al., 1992). BDNF administration in the entorhinal cortex also increased the intensity of synaptophysin immunolabeling in the dentate molecular layer, reversing the age-related decrease in synaptophysin expression in axon terminals within this region.

It is possible that a loss of BDNF production or trafficking causes the age-related degeneration of synaptic structures observed in this study. One previous study reported that BDNF expression declined throughout the hippocampus and entorhinal cortex (excepting CA2) with advanced age in macaque monkeys (Hayashi et al.,

2001). However, in preliminary studies, our group has not detected a change in BDNF protein levels by ELISA in the entorhinal cortices of aged rats (unpublished data). It remains possible that the provision of supra-normal levels of BDNF by lentiviral vector administration may compensate either for age-related losses in endogenous BDNF expression, or may further stimulate neurons already exposed to adequate levels of BDNF.

The reversal of age-related decrements in spine density and morphology was specific to BDNF. Lentiviral vector-mediated NGF gene delivery to the aged entorhinal cortex did not generate changes in spine parameters compared to the control LacZ vector. While other studies have reported NGF-induced increases in terminal branch spine density on cortical pyramidal neurons in adult (Kolb et al., 1997) or aged (Mervis et al., 1991) rats, NGF in the previous studies was infused into the cerebral ventricles, thereby potentially broadly activating cholinergic projections to the cortex. In contrast, our local intraparenchymal injections of lentiviral vectors restricted NGF availability to local neurons. While this local availability was sufficient to stimulate spine parameters using the BDNF gene, NGF had no effect. Thus, the observed trophic effects are specific to BDNF.

E. Conclusion

Normal aging in the hippocampal formation is not typically associated with extensive neuronal death (Amaral, 1993; Rapp and Gallagher, 1996; Merrill et al., 2000; Merrill et al., 2001). Instead, aging is associated with declines in the functional state of neurons (Barnes, 1979; Deupree et al., 1991; Moore et al., 1993; Rosenzweig et al., 1997; Barnes et al., 2000), perturbations in gene expression (Kitraki et al., 1993; Kishimoto et al., 1998; Vela et al., 2003; Gavilán et al., 2007), and significant involutional changes in the functional unit of synapses, dendritic spines. In this study, we have utilized exogenous BDNF expression to reverse synaptic degeneration, a key component of age-related entorhinal neuronal dysfunction. Lentivirus-mediated BDNF expression generated structural plasticity in aged cortical neurons and returned spine density and morphology to levels in young rats.

F. Materials and Methods

Surgery and Cell Filling

Three weeks before cell filling, aged (21-24 months) male rats received intraparenchymal injections of BDNF-expressing, NGF-expressing, or LacZ-

expressing lentiviral vectors (lvv) targeting the dorsomedial entorhinal cortex at 4 bilateral sites (8 sites total). These sites were measured from the rostral boundary of the left and right transverse sinuses for the anterior/posterior coordinates, from lambda for the medial/lateral coordinates, and from the surface of the brain for dorsal/ventral coordinates (Site 1: A/P = +1.1 mm, M/L = ±4.4 mm, and D/V = -3.4 mm; Site 2: A/P = +1.1 mm, M/L = ±4.7 mm, and D/V = -3.4 mm; Site 3: A/P = +0.8 mm, M/L = ±4.4 mm, and D/V = -2.4 mm; Site 4: A/P = +0.8 mm, M/L = ±4.7 mm, and D/V = -2.4 mm). Two micro-liters of lentiviral vector (100 ug/mL p24) were injected at each site.

The same surgical protocol was used on an additional group of aged rats (n = 12) used for synaptophysin analysis. These rats were injected with either BDNF-expressing (n = 6; aged-BDNF) or EGFP-expressing (n = 6; aged-EGFP) lentiviral vector.

Three days before cell filling, all young (4-6 months of age) and aged (21-24 months of age) rats received 4 injections (150 nL per injection) of red fluorescent microbeads (Retrobeads, Lumafluor Inc., Naples, FL) into the rostral dentate gyrus for retrograde labeling of layer II neurons in the dorsomedial entorhinal cortex. Bilateral coordinates were measured from bregma and the surface of the brain (Site 1: A/P = -2.8 mm, M/L = ±1.1 mm, and D/V = -4.2 mm; Site 2: A/P = -3.3 mm, M/L = ±1.7 mm, and D/V = -3.9 mm).

The rats were transcardially perfused using a protocol optimized for cell filling (Mead and Tuszynski, 2008). To produce sections of the dorsomedial entorhinal cortex containing complete layer II neurons, each hemisphere was individually blocked perpendicular to the medial entorhinal cortex pial surface. The blocks were cut to 200 μm-thick sections and collected in ice-cold 0.1 M PB. Neurons were identified for cell filling based on the presence of red fluorescent microbeads. Labeled neurons were impaled with a sharp micropipette and filled by iontophoresis of 5% aqueous solution of lucifer yellow (LY; Sigma-Aldrich Co., St. Louis, MO) using a current of 0.5-5 nA for 1-5 min.

Imaging and Analysis of Spine Density and Morphology

Neurons selected for imaging and analysis were modified spiny stellate cells typical of the excitatory layer II neurons in this region (Fig. 2A; Mikkonen et al., 2000) Dendrite branch segments (Fig. 2B) were collected as z-stack images using a multi-photon microscope with a Ti:sapphire laser tuned to 900 nm (FV300, Olympus America Inc., Melville, NY; Mai Tai IR laser, Newport Co., Mountain View, CA). Branches selected for imaging and analysis consisted of first-degree through fourth-degree apical and second-degree through sixth-degree basilar branches longer than 10 μm. Sub-micron resolution of dendritic spines was obtained with a 60x objective, 5-7x

digital zoom, and 0.1 μm step size. A median filter was used on each image stack prior to analysis.

Spine density (spines/μm dendrite length) was analyzed using maximal intensity two-dimensional (2D) projections from 3D z-stack images. Spine density was calculated for each branch, and the average spine density of each neuron was determined. Spines were then divided into three categories based on morphological features described in previous studies (Harris et al., 1992; De Simoni et al., 2003; Tyler and Pozzo-Miller, 2003). Figure 2C provides a schematic representation of the categories: stubby (type I), mushroom (type II), and thin (type III). Stubby spines had no apparent neck and a length that was no greater than their width. Mushroom spines had a large head with a diameter more than twice their neck diameter. For thin spines, the ratio of head to neck diameter was less than 2. For the cases in which the spine neck was indistinct, the maximum head diameter for thin spines was set at 0.675 μm (spherical volume: 0.16 μm^3). Filopodia, unstable and infrequent spines, were identified as longer protrusions having a greater length than width and lacking a clearly defined head (not shown). Spine type densities were calculated for each branch. Additionally, the proportion of total spines represented by each spine type was determined for each branch.

Histological Analysis

Immunolabeling in a separate group of rats was used to confirm correct targeting, expression, and spread of BDNF, NGF, and LacZ lentiviral vectors (Fig. 1). Lentivirus-treated rats were transcardially perfused with a 2% paraformaldehyde/0.2% parabenzoquinone cocktail, and the brains were cut to 40 μm-thick sections. Light-level immunohistochemistry was performed for BDNF (1:1000; Abcam), NGF (1:1000; courtesy of J. M. Conner), or LacZ (1:1000; Chemicon).

Fluorescent immunolabeling for synaptophysin analysis was performed using a similar protocol. Young (n = 6), aged-EGFP (n = 6), and aged-BDNF rats were transcardially perfused with a 2% paraformaldehyde/0.2% parabenzoquinone cocktail, and the brains were cut to 40 μm-thick sections. Synaptophysin immunohistochemistry was performed with 1:200 dilution of the monoclonal antibody (Chemicon) followed by incubation with a fluorescent secondary antibody (1:200; Molecular Probes).

Synaptophysin quantification was performed on immunolabeled sections from young, aged-EGFP, and aged-BDNF groups. Briefly, using a laser scanning confocal microscope (MRC1024; Bio-Rad) and 200x magnification, three fields per section were randomly selected in each of the target areas (middle, inner, and outer molecular layers of the dentate gyrus). Images were collected in three sections from each animal

and analyzed using NIH ImageJ software (version ImageJ 1.37v; Wayne Rasband, NIH, Bethesda, MD). Uniform thresholding values were used between subjects for quantification of synaptophysin density and intensity. Synaptophysin density was measured as the percent of each field occupied by synaptophysin-immunoreactive presynaptic terminals. Synaptophysin intensity in each field was measured relative to threshold as arbitrary intensity units. Density and intensity measurements from each target area were averaged across fields for each subject and are presented as mean ± standard error of the mean (SEM).

Statistical Analysis

All analyses were performed blinded. The group differences in average spine density, spine type percentage, and synaptophysin density and intensity were evaluated with 1-way ANOVA and Fisher's probable least-squares difference (PLSD) post hoc test. Data are presented as mean ± SEM.

G. Figures

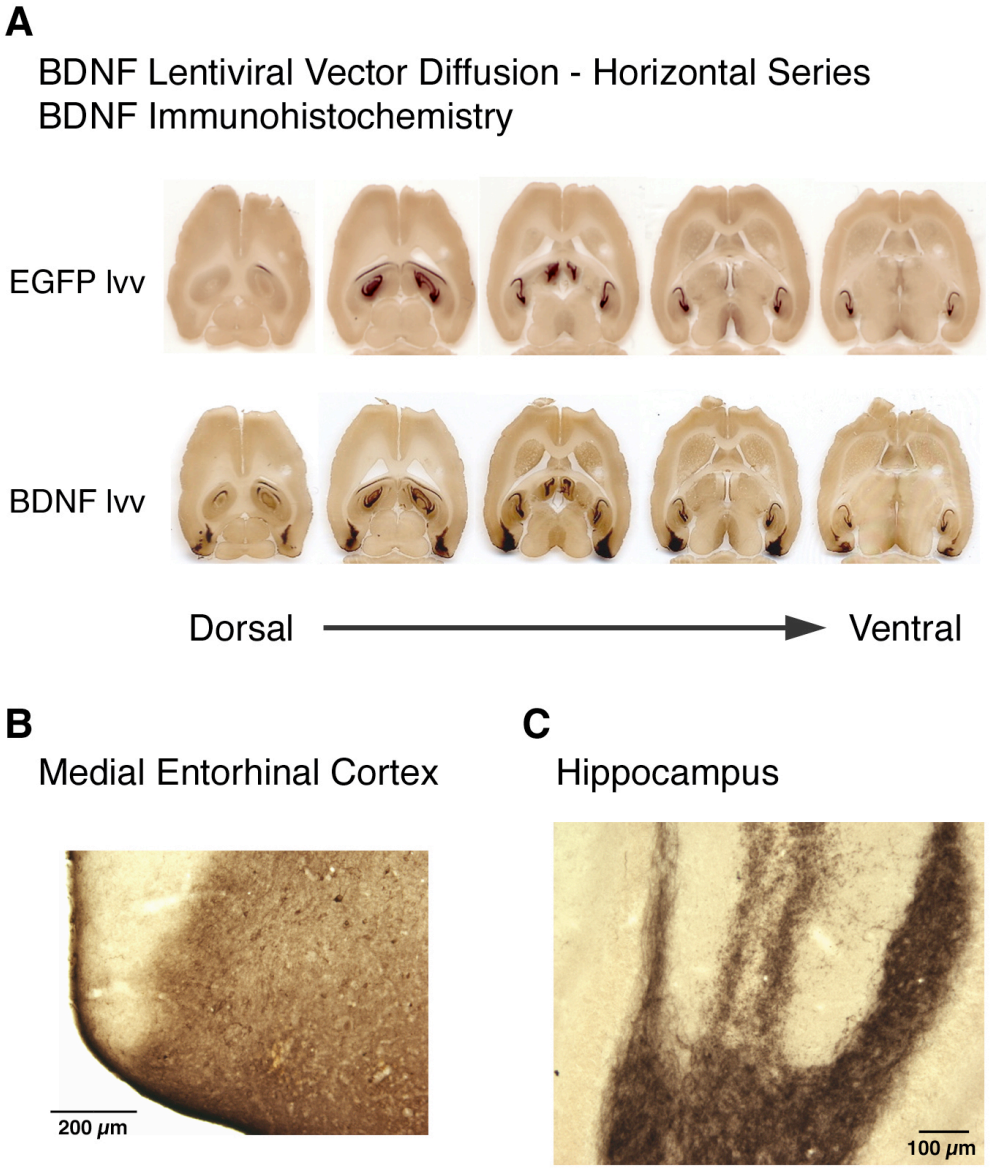

Figure 5. Light-level BDNF immunohistochemistry. **(A)** Serial horizontal sections from two aged rat brains. The first was injected with a control lentiviral vector expressing EGFP. The second demonstrates the targeting and spread of the BDNF lentiviral vector in the dorsomedial entorhinal cortex. Endogenous BDNF expression in the hippocampus is clearly visible in both animals. **(B)** 10x magnification of the right-hemisphere medial entorhinal cortex from section 4 of the "BDNF lvv" horizontal series. **(C)** 10x magnification of the right-hemisphere dentate gyrus and CA3 from section 4 of the "BDNF lvv" horizontal series.

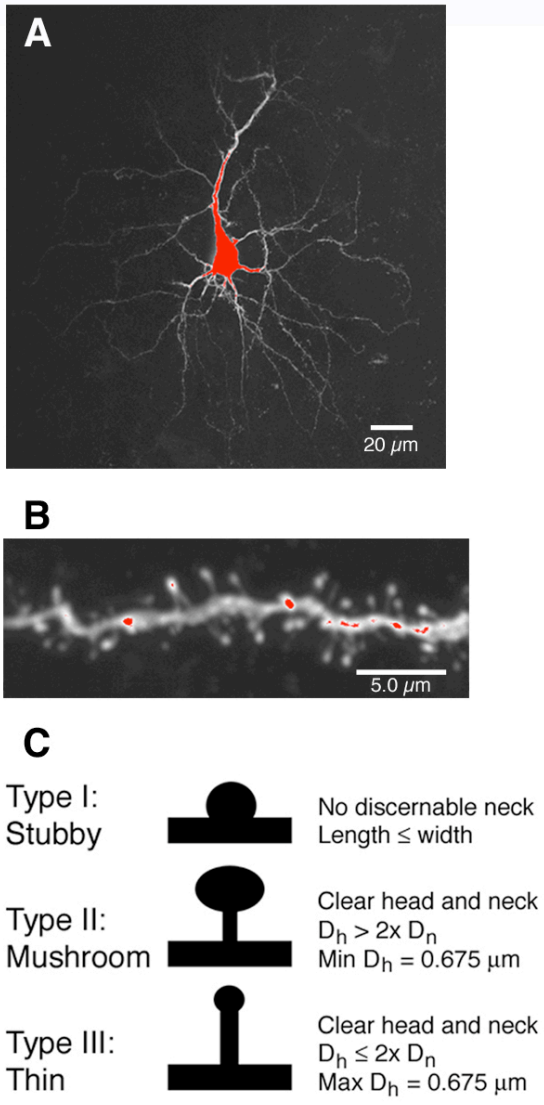

Figure 6. (A) LY-filled layer II neuron from the dorsomedial entorhinal cortex. The neurons filled in this region typically had a modified spiny stellate neuron morphology. **(B)** Maximum intensity projection of a dendrite branch used for spine density analysis. **(C)** Schematic representation of spine categories. Spines were individually classified based on the presence of a neck, the length of the spine relative to its width, and the diameter of the spine's head (Dh) relative to the diameter of its neck (Dn).

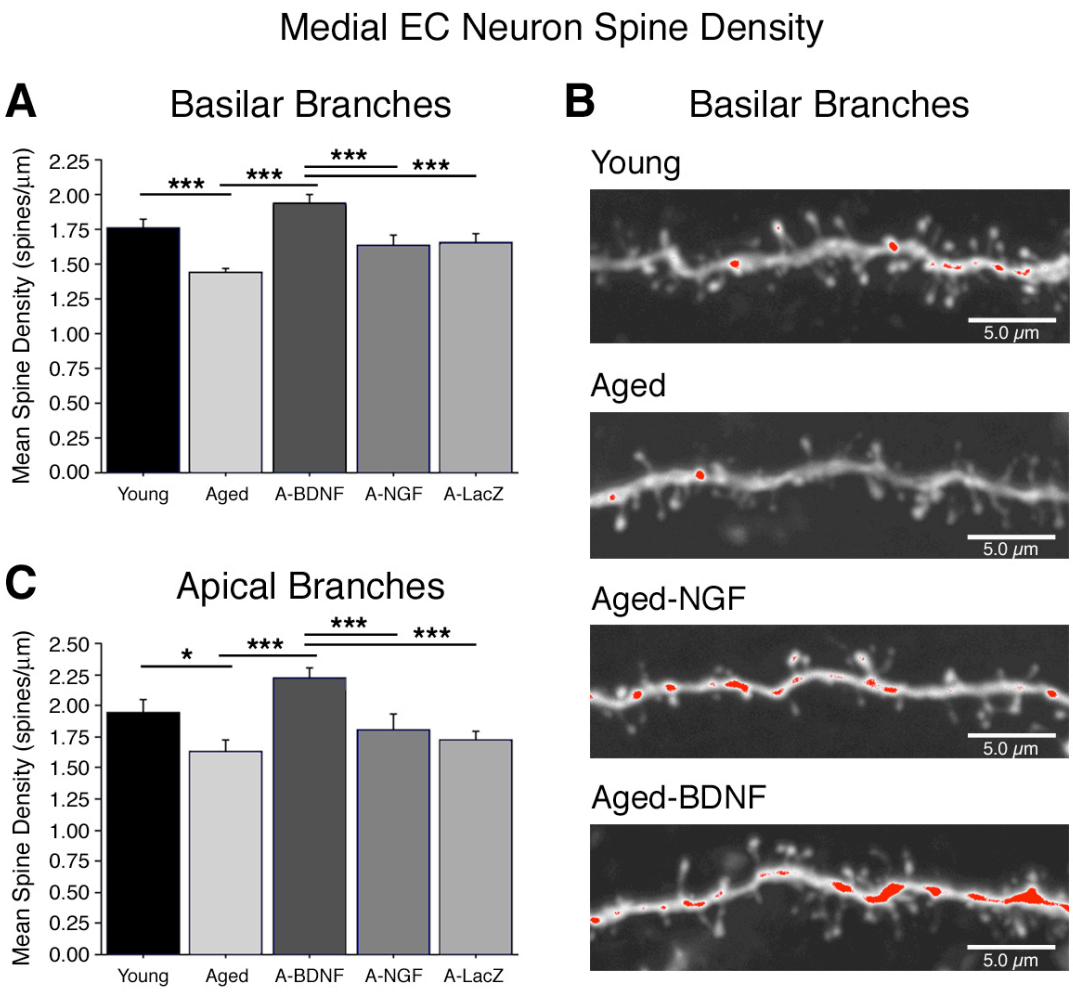

Figure 7. Lentiviral vector delivery of BDNF to aged medial entorhinal cortex neurons reverses age-related spine density decrease on both basilar and apical dendrites. **(A)** On basilar dendrite branches, spine density decreased with age. BDNF lentiviral vector administration increased aged rat spine density. **(B)** Representative images of basilar dendrite branches from young, aged, aged-NGF, and aged-BDNF rats. **(C)** Spine density also decreased with aging on apical dendrites. Exogenous BDNF expression reversed age-related decline in apical branch spine density. * $P < 0.05$, *** $P < 0.001$.

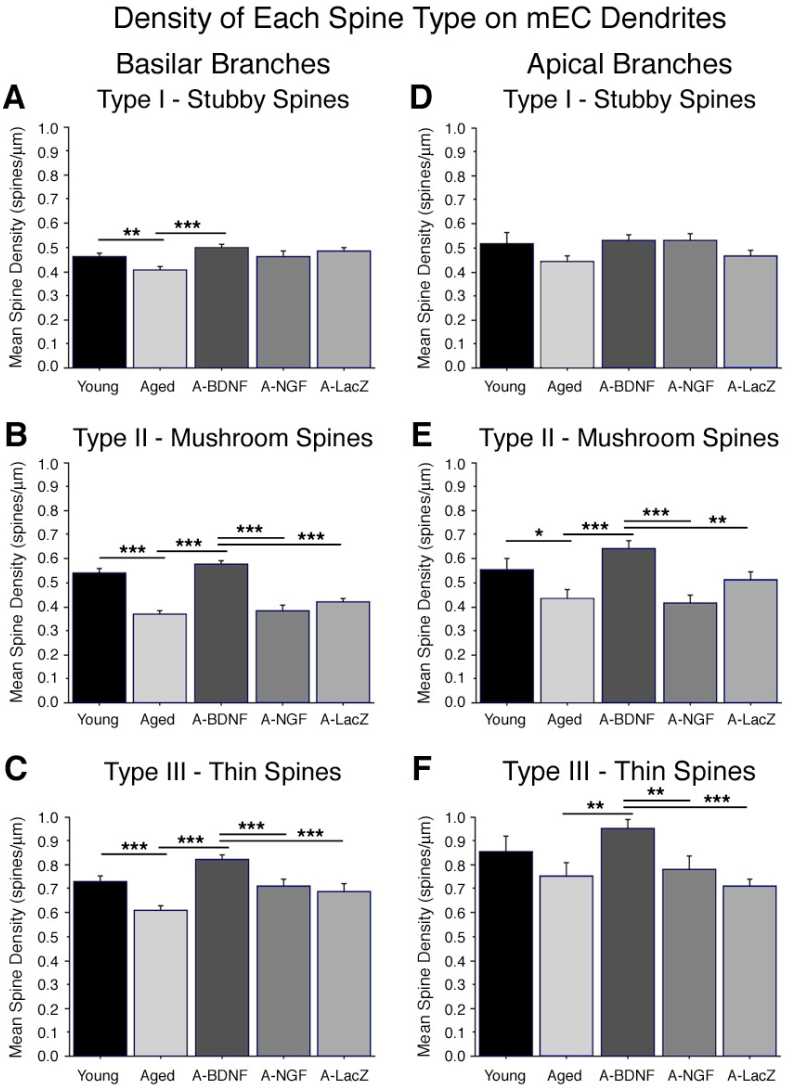

Figure 8. Exogenous BDNF expression increases the density of mushroom and thin spines on aged medial entorhinal cortex neurons. **(A, D)** The density of stubby spines on basilar branches decreased with age, but this change was not significant on apical branches ($P = 0.09$). Although BDNF increased the aged-neuron stubby spine density on basilar branches, the effect likely resulted from the virus injection rather than from BDNF expression. The density measured in the aged-BDNF group did not differ from that of the aged-LacZ control group ($P = 0.50$). **(B, E)** The density of mushroom spines decreased with aging on both basilar and apical dendrites. BDNF increased the aged-neuron mushroom spine density on basilar and apical branches. **(C, F)** Thin spine density showed a significant age-related decrease only on basilar branches. The difference between the young and aged groups did not reach significance on apical branches ($P = 0.17$). BDNF expression increased aged-neuron thin spine density on both basilar and apical branches. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

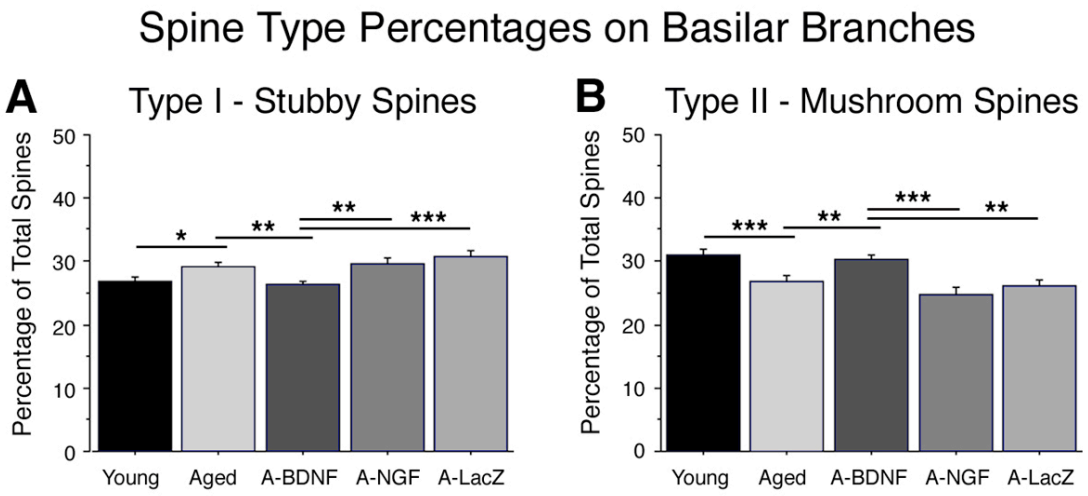

Figure 9. Lentiviral vector delivery of BDNF to aged medial entorhinal cortex neurons reverses age-related changes in spine type percentages for stubby and mushroom spines on basilar dendrites. **(A)** The percent of the spine population represented by stubby spines increased with aging, and this change was reversed by BDNF gene delivery to the aged neurons. **(B)** The percent of the spine population represented by mushroom spines decreased with aging. Exogenous BDNF expression in the medial entorhinal cortex reversed this age-related decline. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

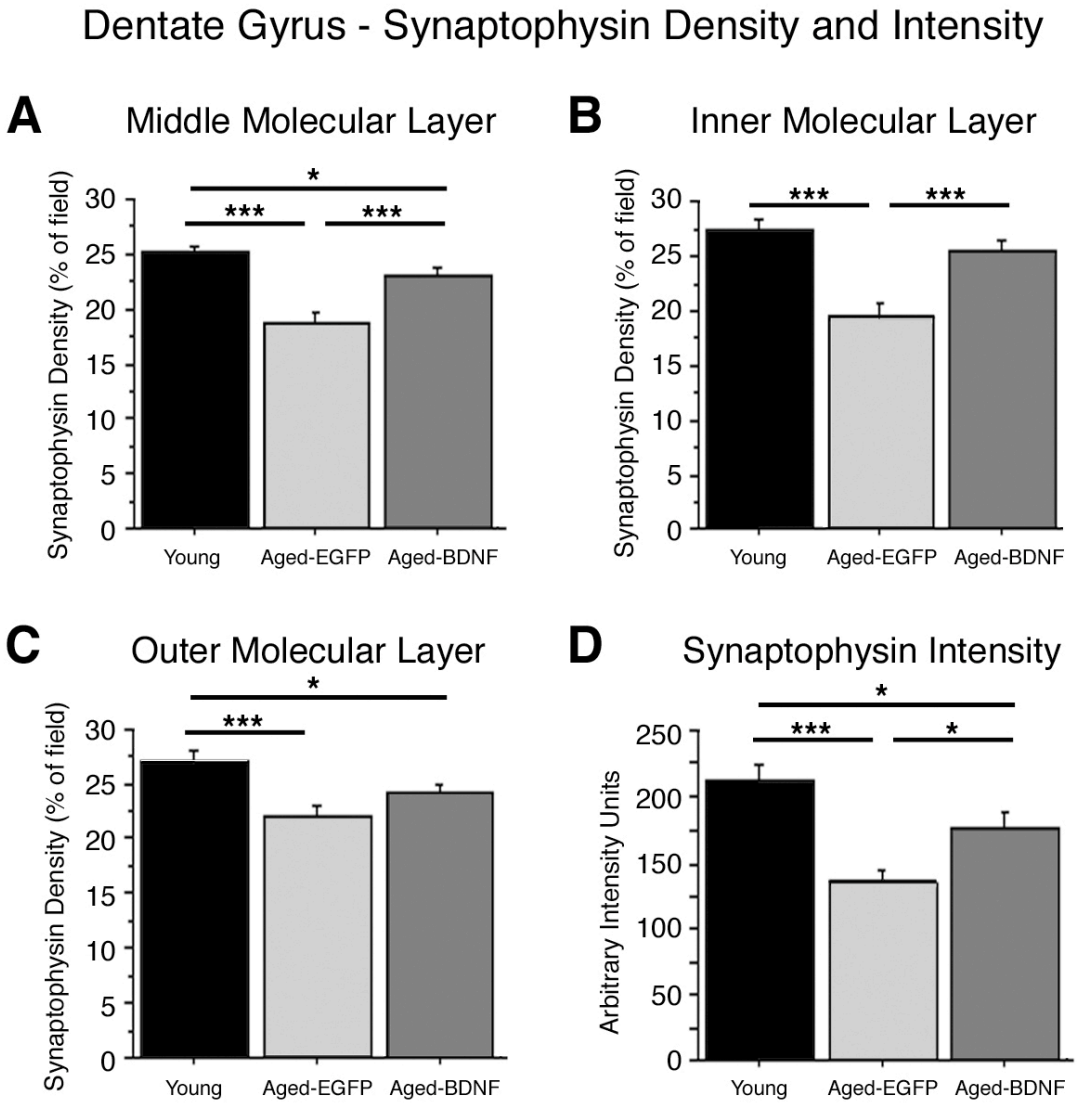

Figure 10. BDNF gene delivery to the medial entorhinal cortex reverses age-related loss of synaptophysin-positive terminals in the dentate gyrus. **(A and B)** BDNF administration significantly increases synaptophysin density in the middle and inner molecular layers of the dentate gyrus. **(C)** Aging results in a significant decline in synaptophysin density in the outer molecular layer. The effect of BDNF on this layer does not reach significance ($P = 0.11$). **(D)** The intensity of synaptophysin labeling in the dentate molecular layer decreases significantly with aging. BDNF administration significantly increases synaptophysin intensity, partially reversing the age related effect. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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I. Chapter Acknowledgements

Chapter 3, in full, is in preparation for publication. Tonya Mead, Eliezer Masliah, Armin Blesch, and Mark Tuszynski are the authors of this manuscript. The dissertation author was the primary investigator and first author of this paper.

IV

BDNF Gene Delivery to the Aged Entorhinal Cortex Reduces Tetanus-Evoked Synaptic Potentiation in the Dentate Gyrus

A. Abstract

Long-term potentiation (LTP) offers a functional model for synaptic plasticity. In the dentate gyrus, traditional LTP induction protocols elicit similar responses from young and aged subjects. The current study replicates these findings. High-frequency induction of LTP in perforant path–dentate granule cell synapses produced equivalent responses in young and aged rats. We then tested the effect of chronic administration of brain-derived neurotrophic factor (BDNF) on perforant path–granule cell synapses in aged rats, using techniques of BDNF gene delivery to the entorhinal cortex. BDNF gene delivery to the aged medial entorhinal cortex led to a significant reduction in high-frequency LTP induction, but did not alter maintenance of the potentiated response. We predict that this change is the result of LTP occlusion and reflects sustained BDNF-induced increases in synaptic activity at aged perforant path–granule cell synapses.

B. Introduction

Long-term potentiation is the amplified response of neurons following a brief set of supranormal stimulus bursts. This potentiation of cellular response persists for hours to days and may demonstrate the limits of the neuron's natural response to normal, memory-encoding synaptic activity. Although many hypothesize that the mechanisms enabling memory encoding in the brain are the same as those that produce tetanus-evoked LTP, it remains unclear whether LTP models memory formation at the cellular level. Nevertheless, LTP provides a model of synaptic plasticity.

The functional plasticity of hippocampal synapses decreases with advancing age (reviewed in Rosenzweig and Barnes, 2003), but can be difficult to assess. In CA1, low-level, perithreshold stimulation reveals age-related deficits in LTP induction (Deupree et al., 1991; Moore et al., 1993; Rosenzweig et al., 1997), whereas traditional high-frequency stimulation (HFS) protocols obscure these effects (Landfield et al., 1978; Deupree et al., 1993; Moore et al., 1993; Diana et al., 1994a,b; Norris et al., 1996). Findings in the dentate gyrus have been less consistent. While several groups have found no age-related changes in LTP induction following high-frequency stimulation (Barnes, 1979; Diana et al., 1994a,b), one group has repeatedly reported impaired induction (Lynch and Voss, 1994; Mullany and Lynch, 1997; Kelly

et al., 2000). As in the CA1 region, LTP induction deficits in the dentate gyrus were more apparent with a less robust stimulation. A protocol combining perithreshold stimulation of the perforant path with granule cell depolarization revealed an age-related increase in LTP induction threshold (Barnes et al., 2000). Although aging is associated with decrements in the functional plasticity of hippocampal synapses, treatments of age-related cognitive decline may improve synaptic function.

Brain-derived neurotrophic factor (BDNF) is an example of a therapeutic agent that may enhance the synaptic function of aged neurons. BDNF, when infused into the medial entorhinal cortex, improved spatial memory in aged rats (Merrill, 2002). Effects of BDNF on synapse function may underlie the observed cognitive improvement. BDNF increases the number of docked vesicles at the active zone of excitatory synapses (Tartaglia et al., 2001), enhances presynaptic glutamate release (Li et al., 1998), and increases NMDA receptor activity (Levine et al., 1998). Furthermore, BDNF administration to hippocampal slices can facilitate LTP induction in dentate granule cells when coordinated with weak, subthreshold stimuli (Kovalchuk et al., 2002), and BDNF in vivo infusion into the dentate gyrus can induce transcription-dependent, late-phase LTP at perforant path–granule cell (PP-GC) synapses (Messaoudi et al., 1998; Messaoudi et al., 2002).

We recently determined that BDNF lentiviral vector administration in the dorsomedial entorhinal cortex (EC) reversed age-related structural changes to medial

perforant path-projecting neurons (Chapter 3). This BDNF expression paradigm may also enhance the function of aged perforant path–granule cell synapses. In cortical neurons, BDNF undergoes anterograde transport and activity-dependent transfer to postsynaptic neurons (Kohara et al., 2001). The vesicular packaging of BDNF (Salio et al., 2007) suggests its transfer to postsynaptic neurons is mediated by synaptic release. Although many studies have shown that BDNF administration modifies local synaptic activity (Kang and Schuman, 1995; Akaneya et al., 1997; Messaoudi et al., 1998; Messaoudi et al., 2002; Kovalchuk et al., 2002), to our knowledge, this study provides the first demonstration that increasing BDNF expression in one region can modify the functional plasticity of another.

This study tested the hypothesis that BDNF gene delivery to the dorsomedial entorhinal cortex in aged rats, and subsequent increase in BDNF expression, can influence the functional plasticity of synapses between EC perforant path-projecting neurons and dentate granule cells. We now find that high-frequency stimulation of the medial perforant path does not evoke age-related changes in LTP induction or maintenance in the dentate gyrus, and that chronic BDNF lentiviral vector administration to the dorsomedial entorhinal cortex decreases tetanus-evoked LTP in the dentate gyrus.

C. Materials and Methods

Subjects

Male Fischer 344 rats were obtained from the Harlan/NIA rodent colony. Four young rats (4 months old) and 11 behaviorally characterized aged rats (24 months old) were used in this study. All aged rats, prior to surgery, showed significant spatial memory impairment on the Morris water maze based on established criteria (Chapter 2). The 11 aged rats were split into one group of 5 that received injections of BDNF-expressing lentiviral vector in the dorsomedial entorhinal cortex, and one group of 6 rats that received injections of EGFP-expressing lentiviral vector. The young, aged-BDNF, and aged-EGFP groups were tested for differences in LTP induction and maintenance using a high-frequency induction protocol. Animal care procedures strictly adhered to the National Institutes of Health *Guidelines for the Care and Use of Experimental Animals* and were approved by the Institutional Animal Care and Use Committee at the San Diego VA Medical Center.

Surgery

Four to five weeks prior to in vivo electrophysiology, aged rats received intraparenchymal injections of either BDNF-expressing or EGFP-expressing lentiviral vector targeting the dorsomedial entorhinal cortex at 4 bilateral sites (8 sites total). These sites were measured from the rostral boundary of the left and right transverse sinuses for the anterior/posterior coordinates, from lambda for the medial/lateral coordinates, and from the surface of the brain for dorsal/ventral coordinates (Site 1: A/P = +1.1 mm, M/L = ±4.4 mm, and D/V = -3.4 mm; Site 2: A/P = +1.1 mm, M/L = ±4.7 mm, and D/V = -3.4 mm; Site 3: A/P = +0.8 mm, M/L = ±4.4 mm, and D/V = -2.4 mm; Site 4: A/P = +0.8 mm, M/L = ±4.7 mm, and D/V = -2.4 mm). Two microliters of lentiviral vector (100 ug/mL p24) were injected at each site.

In vivo electrophysiology

The rats were anesthetized with urethane (1.5 g/kg i.p.) and placed in a stereotaxic frame. A window was opened in the skull to allow access to the perforant path and hippocampus. A bipolar stimulating electrode was then placed in the medial perforant path (8.1 mm posterior to and 4.0 mm lateral from Bregma, and 2.1 mm

below the brain surface), and the recording electrode was slowly lowered into the dentate hilus (3.4 mm posterior to and 1.9 mm lateral from Bregma). The correct depth for the recording electrode was determined by finding the largest positive-going field excitatory postsynaptic potential (fEPSP), approximately 150 to 200 μm below the fEPSP polarity reversal in the granule cell layer. Long-term potentiation was induced in the perforant path–granule cell synapses using high-frequency stimulation (HFS). The pulse amplitude for tetanic stimulation was determined from the input/output curve (I/O curve) as the current that produced 66% of the maximum fEPSP slope (S max). The HFS tetanus was given as a burst of 15 pulses (0.1 msec/pulse) at 400 Hz. The burst was given 8 times with a 10 second inter-burst interval. The post-tetanus stimulus response was measured every 3 minutes for 2 hours.

Statistical Analysis

LTP in the perforant path-granule cell synapses was measured as the slope of the fEPSP normalized to the baseline fEPSP slope. The normalized LTP measurements are given as the mean ± standard error of the mean (Fig. 1). Repeated-measures ANOVA and Fischer's probable least-squares difference (PLSD) post hoc test were used for statistical analysis of group effects. Differences in LTP induction between groups were determined from responses in the 15 minutes following the

initial post-tetanus spike. LTP maintenance was based on two measurements: the responses from the last 15 minutes of testing and the response slope for the 3-hour testing period.

Immunohistochemistry

Following electrophysiology, immunolabeling was used to confirm correct targeting, expression, and spread of the BDNF and EGFP lentiviral vectors in each rat (Fig. 2). The rats were transcardially perfused with a 2% paraformaldehyde/0.2% parabenzoquinone cocktail, and the brains were cut to 40 μm-thick sections. Light-level immunohistochemistry for BDNF or EGFP was performed using 1 μg/ml rabbit polyclonal BDNF antibody (ab6201; Abcam, Cambridge, MA) or 0.33 μg/ml goat polyclonal GFP antibody (Rockland Immunochemicals, Inc., Gilbertsville, PA).

D. Results

In this study, we examined whether perforant path–dentate granule cell synapses exhibit an age-related decline in HFS-evoked LTP. Additionally, we investigated whether BDNF gene delivery to the aged entorhinal cortex increases HFS-induced potentiation in perforant path–granule cell synapses.

HFS-LTP is unchanged by normal aging

Age-related decreases in hippocampal LTP induction and maintenance have been found using perithreshold LTP induction protocols (Deupree et al., 1991; Moore et al., 1993; Rosenzweig et al., 1997), but are not readily observable with high-frequency stimulation (Moore et al., 1993; Diana et al., 1994a,b). Our observations support the results from these previous experiments. In this study, we observed equivalent responses to HFS-evoked LTP in the young and aged-EGFP groups. Post-tetanus responses showed an overall group effect (repeated measures ANOVA: $P < 0.05$), and post-hoc analysis indicated no difference between the young and aged-EGFP groups for the complete 3-hour testing window (Fig. 1; $P = 0.26$). Additionally, the perforant path–granule cell synapses in these groups showed no difference in HFS-evoked LTP during the first ($P = 0.38$) or last ($P = 0.51$) 15 minutes of testing. The potentiated responses of the young and aged-EGFP groups were similarly maintained over the 3-hour testing window; both groups had slightly declining responses with comparable slopes of –0.0010 for the young group and –0.0007 for the aged-EGFP group. These results indicate that neither LTP induction nor maintenance declines with aging in this paradigm.

BDNF gene delivery decreased LTP induction in aged rats

BDNF lentiviral vector delivery the dorsomedial entorhinal cortex of aged rats caused a significant decrease in tetanus-evoked LTP in perforant path–granule cell synapses (Fig. 1). The post-tetanus response of the aged-BDNF group differed significantly from the young group across the complete 3-hour testing window (P = 0.014), and the difference between the aged-BDNF and aged-EGFP groups approached significance (P = 0.078). The evoked response during the initial induction phase was significantly lower in aged-BDNF rats relative to young rats ($P < 0.05$). The induction-phase comparison between the aged-BDNF and aged-EGFP groups approached significance (P = 0.073). A similar difference between groups continued throughout the 3-hour test period. In the last 15 minutes, the response of the aged-BDNF group differed significantly from both the young ($P < 0.05$) and aged-EGFP (P = 0.05) groups. Although BDNF decreased HFS-evoked potentiation in the aged rats, maintenance of the potentiated response was unaffected. The response slope for the 3-hour testing period was comparable for all three groups (young = -0.0010; aged-EGFP = -0.0007; aged-BDNF = -0.0015).

E. Discussion

The importance of this study is three-fold. First, this study provides further evidence that aged and young perforant path–granule cell synapses respond equally to LTP-inducing high-frequency stimulation. Second, to our knowledge, this study is the first to demonstrate that BDNF can influence functional plasticity in a region of the brain separate from where it originates (acting through anterograde transport). Finally, we find that chronic administration of BDNF to the entorhinal cortex influences the functional plasticity of synapses in the dentate gyrus of aged rats.

There are several potential explanations for the observed decrease in tetanus-evoked LTP at the perforant path–granule cell synapse following chronic BDNF lentiviral vector (lvv) administration. First, it is possible that BDNF expression resulted in biochemical toxicity or excitotoxicity in the aged rat entorhinal cortex. Observational evidence suggests this is not the case. Nissl-stained sections from BDNF lvv-injected rats show no evidence of tissue damage to the entorhinal cortex or hippocampus (data not shown). Furthermore, BDNF lentiviral vector administration in aged rats caused no obvious damage (e.g., cell body atrophy) in dye-filled neurons of the medial entorhinal cortex (Chapter 3). Finally, BDNF infusion generated improvements in learning and memory in a previous study, undermining the possibility that damage to this brain region occurred.

A second possibility is that BDNF lentiviral vector expression may decrease synaptic contacts between entorhinal neurons and dentate granule cells through mobilization of previously stable synapses. In an in vitro study, BDNF overexpression in cortical neurons decreased their spine stability (Horch et al., 1999). In our paradigm, measurements of spine density and structure in the entorhinal cortex do not indicate a BDNF-induced decline in stable synapses (Chapter 3). On the contrary, BDNF expression increased the density of highly active and stable mushroom spines on aged entorhinal neurons (Chapter 3). Therefore, it is unlikely that the BDNF expression in this paradigm disrupts stable and active perforant path–granule cell synapses in the aged rats.

Alternatively, BDNF lentiviral vector administration may increase inhibitory contacts with BDNF-expressing neurons. Recent in vitro studies support this possibility. Selective removal of BDNF from a single neuron reduced the number of GABAergic terminals on the cell's soma (Kohara et al., 2007). Relatedly, chronic bath application of BDNF to single-cell GABAergic neuron preparations increased inhibitory autapses (Palizvan et al., 2004). Further research will be needed to clarify whether changes in inhibitory input underlie the BDNF-mediated LTP induction deficit in the current study.

Another possibility is that short-term versus chronic BDNF administration have different effects on the functional plasticity of synapses. While short-term BDNF

delivery enhances synaptic activity, chronic BDNF administration may decrease TrkB signal transduction and undermine synaptic plasticity. In adult rats, chronic BDNF protein infusion into the hippocampus led to a significant reduction in full-length TrkB protein expression and phosphorylation (Frank et al., 1996; Xu et al., 2004). In aged rats, on the other hand, chronic infusion of BDNF into the entorhinal cortex produced a sustained increase in the phosphorylation of Erk, a kinase activated by TrkB signal transduction (Merrill, 2002). These studies suggest that aged and adult neurons react differently to chronic BDNF administration, and predict that chronic BDNF administration in the aged hippocampal formation produces a sustained enhancement of TrkB activation.

The fifth and best-supported possibility suggests that chronic BDNF expression and high-frequency stimulation use overlapping mechanisms to modify perforant path-granule cell synapses. Previous studies have shown that increased potentiation of a particular pathway reduces the amount of subsequent potentiation that can be further evoked in the same circuit (Rioult-Pedotti et al., 2000; Kovalchuk et al., 2002). This phenomenon, called LTP occlusion, indicates that the potential for synaptic enhancement within a neural pathway is limited. In a recent study, Messaoudi and colleagues (2002) demonstrated that BDNF administration in the hippocampus induces late-phase LTP. By inducing LTP with a high-frequency tetanus prior to BDNF administration, they prevented further synaptic enhancement by BDNF.

Similarly, an in vitro study demonstrated that BDNF-facilitated LTP in dentate granule cells prevents further synaptic enhancement by tetanic stimulation (Kovalchuk et al., 2002). In the current study, BDNF-administered aged rats exhibited an attenuated LTP response to high-frequency stimulation. If chronic administration of BDNF in the aged entorhinal cortex can produce an on-going enhancement of the perforant path-granule cell synapses, it could limit the amount of LTP produced by subsequent tetanic stimulation. Thus, the results of the current study may reflect BDNF-induced chronic potentiation of perforant path–granule cell synapses and partial occlusion of subsequent tetanus-evoked LTP. However, studies of BDNF-related LTP occlusion have only studied short-term effects; it remains unclear whether chronic BDNF administration can produce similar results.

Overall, the findings of the present study support the hypothesis that BDNF significantly influences the plasticity of aging circuits in the hippocampal formation. Further studies are needed to confirm that the decrements in tetanus-induced LTP following chronic BDNF administration actually reflect an enhancement of function, as suggested by both morphological analysis (Chapter 3) and behavioral studies. LTP occlusion is a candidate mechanism for the BDNF-induced decrease in tetanus-evoked LTP.

F. Figures

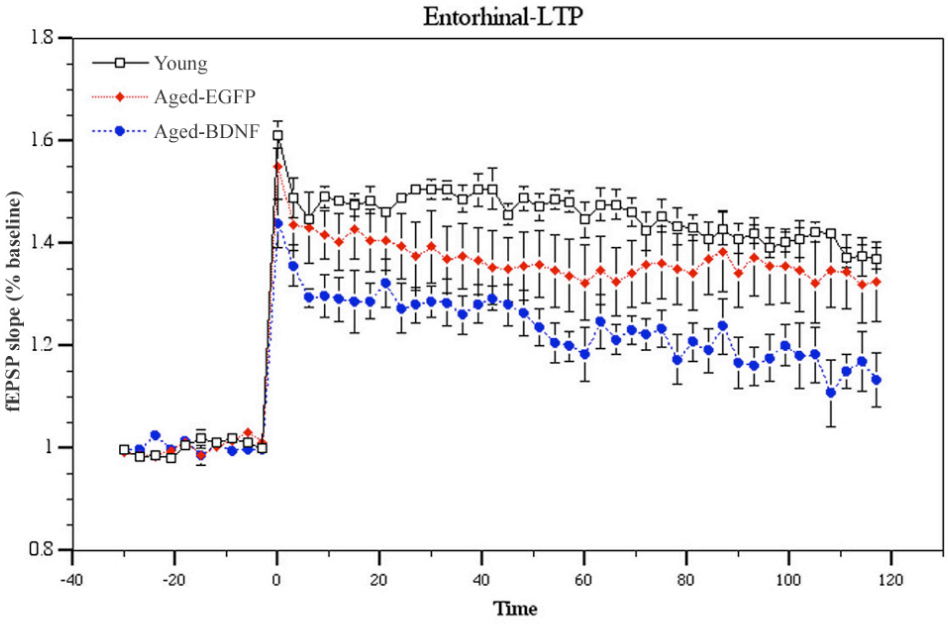

Figure 11. Normalized responses of dentate granule cells before and after high-frequency stimulation of the medial perforant path (time = 0). The groups tested were young (n = 4), aged-EGFP (n = 6), and aged-BDNF (n = 5).

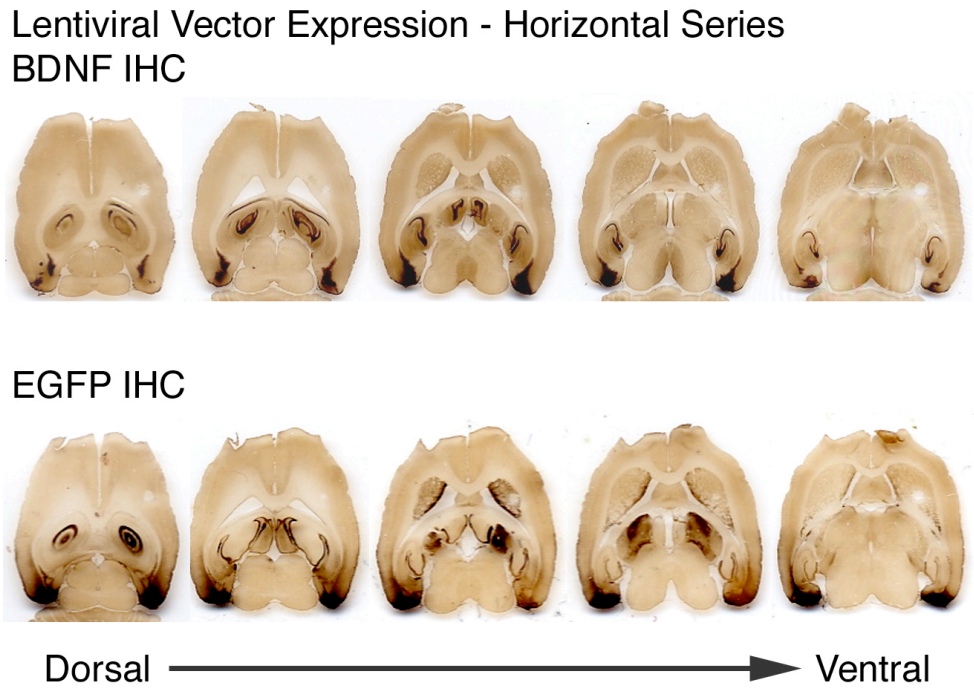

Figure 12. Light-level BDNF and EGFP immunohistochemistry in serial horizontal sections from aged rat brains. These images demonstrate the targeting and spread of the BDNF and EGFP lentiviral vectors in the dorsomedial entorhinal cortex.

G. References

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H. Chapter Acknowledgement

The data in Chapter 4 are the result of the combined efforts of Tonya Mead, James Conner, and Mark Tuszynski.

V

General Discussion

A. Summary of Results

The experiments detailed within this thesis examined age-related changes in synaptic density, structure, and protein expression within the hippocampal formation that may contribute to functional decline. Additionally, these experiments test the sensitivity of synapses in the aged entorhinal cortex and hippocampus to brain-derived neurotrophic factor. More specifically, we have demonstrated that:

- Spine density and morphology decline with aging in the entorhinal cortex. Layer II neurons in the aged rat dorsomedial entorhinal cortex exhibited decreased spine density and a preferential loss of large, mushroom-shaped (Type II) spines. Both changes are consistent with a decline in synaptic function.

- Synaptophysin density and level of expression decreases with aging in the dentate gyrus. The density of synaptophysin-expressing axon terminals decreased

significantly in the middle molecular layer, and throughout the dentate gyrus, in aged rats, suggesting a decline in entorhinal synaptic input to this region. Decreased intensity of the synaptophysin immunolabel additionally suggested that synaptic protein expression decreases in afferent inputs to dentate granule cells.

- Brain-derived neurotrophic factor gene delivery reverses age-related spine density decline in the entorhinal cortex. Layer-II hippocampal-projection neurons in the dorsomedial entorhinal cortex of aged rats increased dendritic spine density in response to BDNF gene delivery. This result suggests that BDNF enhances the connectivity between neurons of the entorhinal cortex and their afferent inputs in aged subjects. However, since not all spines provide stable synapses, we also analyzed spines based on their shape.

- BDNF gene delivery to the aged entorhinal cortex increases the density of all three primary spine types and returns the relative proportions of the different spine types to normal. BDNF lentiviral vector delivery to the dorsomedial entorhinal cortex restored the density of stubby, mushroom, and thin spines to levels comparable to those measured in young subjects. In aged rats, the percentage of stubby spines in the total spine population increased, whereas the relative proportion of mushroom spines decreased. BDNF reversed these age-related changes in spine type proportions. These

results indicate that BDNF increases stable and active excitatory synapses in the aged entorhinal cortex.

- BDNF gene delivery to the aged entorhinal cortex increases the density and intensity of synaptophysin expression in the dentate gyrus. The BDNF-induced increase in synaptophysin density was observed in the middle molecular layer, the primary terminal zone of layer-II entorhinal neurons, and the inner molecular layer of the dentate gyrus. These results suggest that BDNF can reverse an age-related decrease in perforant path–dentate granule cell synapses and enhance synaptic protein synthesis in afferents to the dentate gyrus.

- BDNF gene delivery to the age entorhinal cortex modifies the activity of perforant path–dentate granule cell synapses. Chronic BDNF administration in the dorsomedial entorhinal cortex of aged rats resulted in a significant decrease in the induction of tetanus-evoked long-term potentiation, but not in maintenance of the potentiated response. Among other possibilities, these results suggest that chronic BDNF delivery to aged neurons may down-regulate TrkB expression and reduce synaptic plasticity. Alternatively, chronic BDNF administration to the aged entorhinal cortex may occlude subsequent tetanus-induced LTP.

Together, these results enhance our understanding of changes in the hippocampal formation that likely contribute to age-related cognitive decline. Furthermore, we have demonstrated that additional trophic support for aged neurons in the hippocampal formation, provided by BDNF gene delivery to the entorhinal cortex, reverses age-related degeneration of synapses in this region and modifies the activity of aged perforant path–dentate granule cell synapses.

B. The Structure/Function Relationship in the Aging Brain

Structural changes in the brain are a common effect of aging. A global decline in white matter integrity reflects a loss of axonal connections throughout the brain (Guttmann et al., 1998; Gunning-Dixon and Raz, 2000; Ge et al., 2002; Head et al., 2004). Gray matter is also affected, and experiences regional decreases in volume (de Toledo-Morrell et al., 2000; Raz et al., 2004; Rodrigue and Raz, 2004). Unlike the extensive gray matter degeneration in Alzheimer's disease (Van Hoesen et al., 1991; Goméz-Isla et al., 1996; Juottonen et al., 1998), minor decreases in gray matter volume with normal aging do not reflect widespread neuron loss (Rapp and Gallagher, 1996; reviewed in Peters et al., 1998; Merrill et al., 2000; Merrill et al., 2001; Rapp et al., 2002). In normal aging, neuron loss is minimal and limited to very few cortical and subcortical regions (Smith et al., 2004). Age-related declines in dendrite extent,

dendrite length and degree of branching, are similarly limited; some brain regions even demonstrate an increase in dendrite extent that is likely a compensatory mechanism (Buell and Coleman, 1981; Connor et al., 1982; Flood et al., 1985; 1987). In contrast to the minimal changes in neuron number and dendrite extent, synapses undergo a substantial age-related decrease in many cortical and subcortical regions. Indeed, we observed an 18% decrease in basilar dendritic spine density in layer II neurons of the aged rat entorhinal cortex. This result implies that excitatory inputs to these neurons are reduced with aging. Similarly, we found a highly significant decrease (ranging from 19-29%) in the density of synaptophysin immunolabeling in the dentate gyrus molecular layers. This measure suggests that, with aging, the density of axon terminals innervating dentate granule cells decreases. Electron microscopy in the dentate molecular layers has produced similar results. In these studies, synapse number decreased with aging in the middle and inner molecular layers (Geinisman et al., 1992). In humans, the loss of postsynaptic spines in the prefrontal cortex (Area 10) approached 50% (Jacobs et al., 1997). Extensive loss of presynaptic and postsynaptic structures may account for the small regional decreases in gray matter volume and may have large functional consequences.

Synapse loss can substantially influence neuronal function. Synapses are critical for information flow in the brain; the formation of new synapses and the functional enhancement of already-present synapses are thought to be processes that

underlie memory formation. The results presented in chapters 2 and 3 demonstrate that synapse loss in the hippocampal formation is a characteristic of normal aging. The age-related loss of synapses in the entorhinal cortex and dentate gyrus likely disrupts the normal functions of these regions, and may contribute to age-related impairment of episodic memory formation.

Changes in synapse structure and composition can also affect synaptic function and, therefore, cognition. For example, an increase in the size of the presynaptic active zone allows for greater vesicular binding and neurotransmitter release (Tyler and Pozzo-Miller, 2001). Postsynaptic spines are the sites of greatest plasticity in the synapse. Dendritic spines are motile structures that actively seek out new connections with nearby axons (Knott et al., 2006). In fact, the age-related spine density decrease in the entorhinal cortex described in chapter 2 may reflect either a deficiency in the spine production of aged neurons or the presence of fewer axons. Dendritic spines are also malleable structures whose size reflects the number of AMPA receptors positioned at the synapse (Matsuzaki et al., 2001), and therefore, its activity. Recent in vivo studies demonstrate that the size of a spine indicates its relative long-term stability, with larger spines having greater stability than smaller spines (Trachtenberg et al., 2002; Kasai et al., 2003; Holtmaat et al., 2005). Our studies revealed that the largest spines, mushroom spines, are the most vulnerable to age-related decline in the entorhinal cortex. Therefore, the synapses that remain may be less active and stable.

Our findings from chapters 2 and 3 support the hypothesis that aging produces synaptic changes in the entorhinal cortex and hippocampus that undermine their normal functions. Although previous studies have investigated synapse loss in the dentate gyrus as a function of normal aging, our studies provide the first evidence that the entorhinal cortex is also affected. The entorhinal cortex is of particular interest in the aged brain because it is an early target for degeneration in Alzheimer's disease (Gomez-Isla et al., 1996; Juottonen et al., 1998), and because it generates the spatial representations necessary for the formation of spatial memory in rodents (Fyhn et al., 2004), a type of episodic memory that frequently declines with aging. Thus, dendritic spine loss in the entorhinal cortex may be a mechanism underlying age-related spatial memory impairment.

C. The Influence of BDNF on Synaptic Structure and Function in Aging

Delivery of brain-derived neurotrophic factor to the medial entorhinal cortex improves spatial memory in aged rats (Merrill, 2002), but the mechanisms responsible for this behavioral improvement have remained unclear. Our aging studies provided a potential target for analysis. In this thesis, we have investigated whether BDNF can reverse the synaptic changes in the hippocampal formation that occur with normal aging.

Neurotrophins modulate synapse structure in the adult brain. BDNF and NGF, in particular, can increase spine density (Tyler and Pozzo-Miller, 2001; 2003; Mervis et al., 1991; Kolb et al., 1997) and BDNF modifies synaptic morphology (Tyler and Pozzo-Miller, 2001; 2003) in mature neurons. Our research determined that local administration of BDNF lentiviral vector increased spine density and influenced spine shape in aged neurons. Local administration of NGF lentiviral vector, on the other hand, had a minimal effect. This difference in the effect of BDNF versus NGF is likely a function of local patterns of receptor expression for the respective growth factors: trkB is expressed by many intrinsic neuronal populations in the entorhinal cortex (including layer II neurons) and by axons projecting into it, whereas NGF is expressed only on the terminals of cholinergic axons (Yan et al., 1997; Tokuyama et al., 1998; Rossi et al., 2002; Prakash et al., 2004). Thus, BDNF may have had a greater influence on the layer II entorhinal cortical neurons that we were sampling. Although previous studies have reported effects of NGF on spine density in frontal, motor, and anterior cingulate cortices (Mervis et al., 1991; Kolb et al., 1997), in these studies, NGF was infused into the lateral ventricles and could thereby directly stimulate the broad extent of basal forebrain neurons. In contrast, we restricted NGF action to local cholinergic terminals in the entorhinal cortex.

BDNF may also have the ability to enhance synaptic function by modifying synapse size. In a previous study, BDNF increased the active zone length on

presynaptic terminals (Tyler and Pozzo-Miller, 2001). Our studies suggest that BDNF can also increase spine size. In the aged entorhinal cortex, BDNF increased the density of all spine types, returning them to the levels measured in young subjects. Additionally, BDNF increased the proportion of large, mushroom spines relative to the total spine population. Although the effect of BDNF on spine dimensions was not directly measured in these studies, the possibility that BDNF increases spine size warrants further investigation. Several cognitive disorders are associated with spine loss, shrinkage, or dystrophy (Fiala et al., 2002); therefore, BDNF gene delivery may have therapeutic benefit beyond its effects on normal aging.

The effect of BDNF on synapse structure was not limited to the entorhinal cortex. BDNF gene delivery to the entorhinal cortex increased the density of synaptophysin labeling in the dentate gyrus indicating a likely increase in synapse density in this region. These changes highlight the ability of neurotrophins to act at a distance from where they are produced. BDNF, in particular, undergoes both anterograde and retrograde transport (see Conner et al., 1998; Tonra, 1999 for review). This means that, in our paradigm, BDNF expressed by cells in the entorhinal cortex may influence wide-ranging brain regions including all of the cortical and subcortical areas that either send projections to the entorhinal cortex, or receive inputs from the neurons found there. Because of its position as a relay for information flow between

the hippocampus and neocortex, the entorhinal cortex provides a useful target for therapeutic administration of BDNF.

In the brain, structure dictates function; therefore, BDNF-induced changes in synaptic structure also reflect changes in synaptic function. To measure these changes, we induced long-term potentiation in the medial perforant path of aged rats using high-frequency stimulation. Chronic BDNF gene delivery to the entorhinal cortex in aged rats produced a significant decrease in tetanus-induced LTP. This result evokes several possible mechanisms. First, chronic administration of exogenous BDNF may induce compensatory changes in the affected neurons to the elevated BDNF levels over time. Whereas short-term BDNF delivery to adult neurons facilitates synaptic activity (Messaoudi et al., 1998; 2002), chronic BDNF administration can down-regulate TrkB expression (Frank et al., 1996; Xu et al., 2004) and may undermine synaptic function. However, aged neurons appear to differ from adult neurons in their responses to chronic BDNF delivery. In aged rats, chronic BDNF delivery to cortical neurons produced a sustained increase in the phosphorylation of ERK, a kinase activated by TrkB signal transduction (Merrill, 2002). This indicates that the aged neuron response to high-levels of BDNF may not be attenuated with time in a chronic expression model.

We have also considered LTP occlusion as a potential mechanism for the observed decrease in tetanus-evoked LTP following chronic BDNF administration.

Previous studies have shown that increased potentiation of a particular pathway reduces the amount of subsequent potentiation that can be further evoked in the same circuit (Rioult-Pedotti et al., 2000; Kovalchuk et al., 2002). This phenomenon, called LTP occlusion, demonstrates that the potential for synaptic enhancement within a neural pathway is limited. Since BDNF alone is able to induce late-phase LTP (Messaoudi et al., 1998; 2002), it may reduce the amount of LTP that can be produced by subsequent stimulation. However, studies of BDNF-related LTP occlusion have only studied short-term effects. Additional investigation is necessary to determine whether chronic BDNF administration in the aged brain can produce similar results.

In addition to its effects on synapse structure and function, BDNF induces gene transcription by modulating the activity of select intracellular signaling cascades (reviewed in Huang and Reichardt, 2003). In fact, BDNF-induced late-phase LTP is dependent on transcription and new protein synthesis (Nguyen and Kandel, 1996). In the present set of studies, we determined that BDNF increased the intensity of synaptophysin immunolabeling in the aged dentate gyrus. This suggests that BDNF increases the synthesis of synaptic proteins in aged neurons. Future studies investigating the effects of BDNF on gene expression and protein synthesis in the aged brain could greatly improve our understanding of the mechanisms through which BDNF reverses age-related synaptic changes in the hippocampal formation.

The studies presented in this dissertation provide a greater understanding of the age-related changes in the hippocampal formation that contribute to cognitive decline in normal aging. The studies also provide insight into the mechanisms engaged by BDNF in the aged hippocampal formation that enable it to improve cognitive function. We have determined that increased trophic support to the hippocampal formation reverses the age-related structural changes in this region and modifies the function of perforant path–dentate granule cell synapses.

D. Future Studies

The studies detailed in this dissertation suggest a number of future experiments. The profile of gene expression changes induced by BDNF in the aged brain would provide us with a greater understanding of the mechanisms that produce the synaptic changes in our studies. A profile of gene expression in the aged entorhinal cortex and hippocampus might also point to sources of age-related synaptic decline in these regions (e.g., mitochondrial degradation or decreased BDNF expression or signaling).

There is also ample opportunity for further exploration of the functional changes in the hippocampal formation with aging. The traditional LTP induction protocols use a strong induction stimulus and frequently mask small age-related

changes (Moore et al., 1993; Diana et al., 1994; Norris et al., 1996). Perithreshold stimulation, on the other hand, has successfully revealed age-related deficits in LTP induction within the hippocampus (Deupree et al., 1991; Moore et al., 1993; Rosenzweig et al., 1997). So far, few have used this technique to investigate age-related changes in the perforant path (Barnes et al., 2000).

In vivo imaging of spines in aged animals is an elegant method for directly measuring changes in these motile structures that would be impossible to measure in our current preparations. This method would allow us to investigate age-related changes in spine motility, such as retraction and extension, and enable us to measure changes in spine turnover. The age-related decrease in spine density measured in our studies may be the result of decreased spine extension, increased spine withdrawal, or a separate mechanism. Each of these possibilities would provide further insight into the source of the age-related changes.

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