UNIVERSITY OF CALIFORNIA, SAN DIEGO

Effects of Brain-Derived Neurotrophic Factor (BDNF) Vector Delivery Into the Entorhinal Cortex on Hippocampal Neurogenesis in Aged Non-Human Primates

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by

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The Thesis of Howard Nguyen is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2013

DEDICATION

I dedicate this thesis to:

My everloving grandmother for the time, love, and effort she put into raising me; for continuing to love and support me; and for always persevering through the toughest of times.

Dad for always being supportive of my decisions, and for teaching me how to think.

Mom for always making time to meet my often unreasonable demands, and for the miraculous cooking.

Truman for the joy and laughter through thick and thin, and for being a continuous source of motivation.

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ABSTRACT OF THE THESIS

Effects of Brain-Derived Neurotrophic Factor (BDNF) Vector Delivery Into the Entorhinal Cortex on Hippocampal Neurogenesis in Aged Non-Human Primates

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To date, there are at least two locations in the mammalian brain where new neurons are being born, one of which is the subgranular zone of the hippocampus. These newborn neurons are fully capable of integrating and functioning as part of the hippocampal circuitry, thereby improving performance on learning and memory tasks. Hippocampal neurogenesis may be modulated by different stimuli and treatments, such as brain-derived neurotrophic factor (BDNF). In the present study, BDNF vector is delivered into the entorhinal cortex. BDNF is anterogradely transported from the entorhinal cortex into the aged non-human primate hippocampus. Newborn cells are detected by use of 5-bromo-2'-deoxyuridine (BrdU). Neurogenesis was measured by the number of BrdU+ cells that colabeled with either a mature neuronal marker (NeuN) or an

immature neuronal marker (DCX). It was determined that while BDNF gene delivery resulted in an increased trend of surviving cells, there was no difference in the number of these newborn cells that differentiate into neurons. While the rate of neurogenesis was unaffected by aging, there was an observed decline in the total number of BrdU+ cells colabeled with a neuronal marker as well as the total amount of DCX+ cells. There was, however, a large portion of the BrdU+ cells that remained unidentified. No BrdU+ cell was colocalized with glial fibrillary acidic protein (GFAP), present in astrocytes and neural progenitor cells (NPCs). Lastly, there was no effect of treatment on maturation of newborn neurons as measured by migration and branching of DCX+ cells.

INTRODUCTION

Alzheimer's disease (AD) is characterized by progressive decline of memory and cognitive ability due to loss of synapses and eventually neuronal death along with the production of β-amyloid plaques (Terry et al., 1964) and neurofibrillary tangles (Kidd, 2006). The entorhinal cortex, one of the first brain regions affected by AD, sends projections into the hippocampus as part of a circuit crucial to learning and memory (Witter et al., 2000). Deterioration of entorhinal cortical neurons leads to cognitive decline and loss of short-term memory, trademarks of AD (Devanand et al., 2007). Hence, identifying novel therapeutic strategies to prevent or attenuate neuronal death in this brain region may be beneficial toward clinical treatment of AD. In particular, brain-derived neurotrophic factor (BDNF) has been described to have some neuroprotective effects in both primates and rodents models of AD (Nagahara et al., 2009). These studies demonstrated that BDNF gene delivery into the entorhinal cortex partially reverses the cognitive decline in both primate and mouse AD models that is suggested to be a result of neuronal atrophy. These findings support the potential therapeutic efficacy of BDNF for ameliorating cognitive decline and neuronal atrophy.

One such approach to utilizing BDNF is by gene therapy; previous work in our lab showed that viral vector-mediated delivery of the BDNF gene to the entorhinal cortex results in anterograde transport of the neurotrophic factor from the entorhinal cortex to the hippocampus, a center critical for cognitive function (Devanand et al., 2007). In this study, AD model rhesus

macaques (Macaca mulatta) received injections of BDNF vector within the entorhinal cortex. BDNF has widespread effects on the central nervous system (CNS). One suggested effect of BDNF is modulating neurogenesis, the birth of new neurons (Benraiss et al., 2001; Pencea et al., 2001; Zigova et al., 1998). Neurogenesis in the hippocampus has been linked to learning and memory tasks in rodents (Deng et al., 2009; Dupret et al., 2008; Garthe et al., 2009; Gould et al., 1999a). To date, many studies have reported neurogenesis in different mammalian models, including primates, although none has examined the effect of BDNF gene delivery on neurogenesis in primates. The overall aim of this study is to investigate whether BDNF gene delivery to the entorhinal cortex may have an effect on hippocampal neurogenesis in the aged primate.

Brain-derived neurotrophic factor

Neurotrophic factors are small proteins secreted by neurons that are widely recognized for their roles in promoting growth and proliferation of cells (Hamada et al., 1996) as well as supporting axonal growth (Martinez et al., 1998), dendritic arborization (Bennett et al., 1998), and synaptic plasticity (Minichiello et al., 1999). The first neurotrophin to be discovered was nerve growth factor (NGF) in 1951 (Levi-Montalcini and Hamburger, 1951). NGF is now classified as part of the 'classic' family of neurotrophins, which also includes BDNF, and NT-3 and 4 (Hohn et al., 1990). BDNF was first purified in 1982 from a pig brain by Yves Barde and Hans Thoenen (Barde et al., 1982). BDNF is translated as a 32-35 kDA pro-isoform; the pro-isoforms are cleaved into mature proteins which

act as ligands for specific receptors (Seidah et al., 1996). Pro-BDNF is packaged into vesicles, which are secreted from the neuron via an activity-dependent process (Goodman et al., 1996; Mowla et al., 1999). Once outside of the neuron, BDNF is cleaved by a protease called plasmin and assumes its mature conformation (Lee et al., 2001; Pang et al., 2004).

Post-synaptically, BDNF binds to receptors tropomyosin-related kinase receptor type B (TrkB), a tyrosine kinase receptor (Soppet et al., 1991), as well as p75, a member of the tumor necrosis factor receptor family (Carter et al., 1996). While mature BDNF binds with the greatest affinity to TrkB, pro-BDNF actually binds more strongly to p75 (Kaplan and Miller, 2000; Klein et al., 1991). BDNF and TrkB are both expressed widely throughout the nervous system, including the cortex, hippocampus, brainstem, and spinal cord (Yan et al., 1997). At the cellular level, both BDNF (Schuman et al., 2006) and TrkB mRNA (Tongiorgi et al., 1997) are localized in dendritic spines.

TrkB can be found in one of three conformations under physiological conditions: as either a full-length receptor or one of two truncated forms; only the full-length receptor has intracellular tyrosine kinase activity (Allendoerfer et al., 1994). When activated, the full-length TrkB receptor homodimerizes, resulting in transphosphorylation and consequently activating downstream signaling pathways (Noble et al., 2011). The truncated TrkB receptors, however, form heterodimers with the full-length receptor as a means of BDNF-activity inhibition (Eide et al., 1996) and sequestering of extracellular BDNF into the cell (Haapasalo et al., 2002).

BDNF and TrkB are involved in development as well as maintenance of neurons (Alderson et al., 1990). It is well understood that BDNF is a critical player in neuronal development and differentiation (Grothe and Unsicker, 1987; Hofer and Barde, 1988; Kalcheim and Gendreau, 1988). For example, with the identification of BDNF by Yves Barde and Hans Thoenen, its role in promoting survival of dorsal root ganglion neurons was also discovered (Barde et al., 1982). BDNF/TrkB interaction has been shown to support neuronal survival and promote expression of anti-apoptotic proteins (Kaplan and Miller, 2000). One experiment showed that mice homozygous null for BDNF were unable to survive past three weeks (Lyons et al., 1999). This may be due in part to the role of BDNF in prevention of apoptosis in the developing nervous system (Bhave et al., 1999; Von Bartheld and Johnson, 2001; Ward and Hagg, 2000). BDNF's developmental effects are widespread, affecting neurons of many different systems, including the dorsal root ganglia (Acheson et al., 1995) to the cortex and hippocampus (Huang and Reichardt, 2001).

In addition to the development of the nervous system, BDNF plays a part in modulating dendritic branches and spine morphology in the matured brain (Tanaka et al., 2008; Vigers et al., 2012). Accordingly, many studies have described BDNF and TrkB as inducing and prolonging long-term potentiation (LTP) (Figurov et al., 1996; Kang et al., 1997; Korte et al., 1995; Patterson et al., 1996). Furthermore, BDNF has been shown to be involved in spatial learning and memory (Figurov et al., 1996; Linnarsson et al., 1997; Tanaka et al., 2008). BDNF depletion by antibody showed a decline in spatial learning in mice (Alonso

et al., 2002). Additionally, knock out of the BDNF gene (Linnarsson et al., 1997) or the TrkB receptor (Minichiello et al., 1999) produced mice with cognitive deficits. Together, these results signify an important role of BDNF in learning and memory formation in the mature brain.

BDNF has also been investigated in relation to aging. It has been reported that BDNF signaling decreases with age; studies have shown decreased expression of both mature BDNF protein (Driscoll et al., 2012; Silhol et al., 2005) and the TrkB receptor (Perovic et al., 2012; Silhol et al., 2005) in aged animals. This loss has been suggested to be an underlying factor for some degree of age-related decline of cognitive function and synaptic plasticity (Endres and Lessmann, 2012; Zeng et al., 2011). Retention of synaptic plasticity in aging models in the hippocampus has been cited to correlate with better memory task performance (Platano et al., 2008). Interestingly, plasticity significantly declines with aging (Burke and Barnes, 2006). Together, this data seem to suggest that the decline in BDNF signaling pathway may underlie cognitive decline in aging models.

Neurogenesis

For much of the history of neuroscience, it was believed that the birth of new neurons in mammals was limited to prenatal development. Formation of new neurons, also known as neurogenesis, in the adult brain was first discovered in 1962 in a lesioned rat brain (Altman, 1962). To date, there are at least two locations within the adult mammalian brain that exhibit neurogenesis: the

subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus located in the hippocampus.

In the adult mammalian subventricular zone, proliferating neural progenitor cells (NPCs) give rise to neuroblasts, which migrate down the rostral migratory stream and into the olfactory bulb (Curtis et al., 2012). In the olfactory bulb, these newly born neurons mature into local interneurons (Altman, 1969; Lois and Alvarez-Buylla, 1994). The nature of this migration is similar in both primates and rats (Doetsch and Alvarez-Buylla, 1996; Kornack and Rakic, 2001). The neural progenitor cells of the SVZ have been shown to express glial fibrillary acidic protein (GFAP), an intermediate filament protein expressed primarily in astrocytes (Doetsch et al., 1999). Previous to this discovery, it was assumed that neuroglia and neurons had different precursors and lineages (Alvarez-Buylla and Garcia-Verdugo, 2002). The ongoing stream of neurons into the olfactory bulb may play an adaptive role for changes in the environment of odors (Alvarez-Buylla and Garcia-Verdugo, 2002). The integration and functional roles of newly born cells in the adult brain are not yet fully understood.

Neurogenesis in the SGZ shares some key features with that in the SVZ but also has its own distinct properties. One such similarity in between these two zones is that GFAP-expressing progenitor cells serve as the primary precursor for new neurons in both zones (Seri et al., 2001). Progenitor cells in the SGZ, however, are far less abundant than in the SVZ (Curtis et al., 2012); studies suggest that this pool of progenitor cells stabilize to a fixed number by adulthood (Boekhoorn et al., 2006; Low et al., 2011; Lucassen et al., 2010). In the macaque,

hippocampal neurogenesis peaks within the first three postnatal months; recent research suggests that a combination of cell proliferation and death leads to a stable population of neurons in the granule cell layer (Jabes et al., 2010). Furthermore, studies have shown that the maturation of new granule cells in the dentate gyrus of the macaque monkey takes at least six months (Kohler et al., 2011). In addition to new neurons, oligodendrocytes and astrocytes have also been reported in the hippocampus of macaques (Kornack and Rakic, 1999). The same study found that rodents expressed tenfold higher amounts of neurogenesis compared to the monkeys. This difference in amounts of neurogenesis may be due to longevity among species rather than body size and mass (Lazic, 2012).

Progenitor cell lineages in the SGZ can be characterized and separated into different cell types. There is a population of neural stem cells that self-renews and gives rise to a pool of neural progenitor cells (NPCs) that stabilizes in adulthood (Gage, 2000). Of the NPCs, there are two subtypes: type 1 are radial astrocytes while type 2 are nonradial (Williams and Lavik, 2009). While type 2 NPCs arise from radial astrocytes, both give rise to intermediate progenitor cells (IPCs) that in turn can give rise to neuroblasts and astrocytes (Ming and Song, 2011; Suh et al., 2007). Progenitor cells can be detected by several markers including Sox-2, a marker of neural stem and progenitor cells, and GFAP, a glial marker (Ming and Song, 2011).

The rate of neurogenesis can be affected by many external stimuli. Recent studies have shown that exercise may have a positive effect on neurogenesis although the mechanism is unknown (Bechara and Kelly, 2013;

Speisman et al., 2013). Enriched environments, with (Mustroph et al., 2012) and without exercise (Birch et al., 2013), have also been shown to impact on neurogenesis in rodents. In addition to these, there have been experiments demonstrating the effects of chemical compounds in the regulation of neurogenesis, including estrogen (Tanapat et al., 1999) and anti-depressants (Malberg et al., 2000). The next section will discuss the current findings on the relationship of BDNF and neurogenesis.

The use of 5'-bromo-2'-deoxyuridine (BrdU) has become a common technique in the assessment of neurogenesis. BrdU is an analog of the nucleic acid thymidine and is incorporated into DNA during replication. Thus, any cell that is undergoing the mitotic S phase at the time of the BrdU injection can be visualized using an antibody probe against BrdU. Incorporation of BrdU will allow visualization of both mother and daughter cells (Wojtowicz and Kee, 2006). It has been shown that BrdU can still be detected two years post-infusion (Eriksson et al., 1998). BrdU labeling may give insight on cell proliferation or cell survival, both of which are measures of neurogenesis. The assessment of cell survival requires at least two different subject survival times based on one BrdU injection. Comparing the amount of BrdU+ cells present at a second time point provides insight on whether or not the cells are surviving. In terms of proliferation, the amount of BrdU+ cells must be assessed immediately after BrdU administration to avoid multiple mitotic cycles. Because tissue was not examined immediately after BrdU administration, the BrdU+ cells in this study do not reflect just proliferation. Rather, BrdU+ cells in the present study reflect both

proliferation and survival. More specifically, the BrdU+ cells represent the amount of proliferative cells that survived. BrdU was administered three weeks post-surgery over a span of 3 days. Subjects survived between 1 and 24 months post-BrdU administration.

BDNF and Neurogenesis

BDNF depletion in the hippocampus has been found to lead to an increase in cell proliferation along with reduced numbers of cells differentiating into neurons (Waterhouse et al., 2012); similarly, selective knockout of the TrkB receptor results in similar findings. There is evidence, however, that indicates BDNF depletion in the hippocampus leads to increased cell proliferation while the number of differentiating cells remains unaffected (Chan et al., 2008). Newborn neurons in BDNF knockout mice, however, are stunted in maturation as shown by the decrease in markers of mature granule neurons such as calbindin. BDNF has also been found to be at least required for basal neurogenesis (Lee et al., 2002); BDNF knockout in mice resulted in both fewer BrdU+ cells and newborn neurons, which is different from previous findings. Lee et al. suggests that BDNF promotes basal neurogenesis by supporting the survival of newly generated hippocampal neurons in mice under dietary restriction. Additionally, a study using environmental enrichment (EE) shows that there are no differences in cell proliferation or differentiation between BDNF-knockout and wild-type mice (Choi et al., 2009); however, in both normal housing conditions and EE, mice with reduced BDNF exhibited impaired survival of newborn neurons. These

studies provide evidence that BDNF modulates neurogenesis in the adult rodent hippocampus; the mechanisms and difference in results, however, are unclear.

Neurogenesis is suggested to be modulated by BDNF through GABAergic interneurons located in the hilus through a series of studies by Waterhouse et al (Waterhouse et al., 2012). Reduction of BDNF transcript in the granule cells lead to an increase in cell proliferation, but a decrease in neurogenesis. Evidence suggests this is due to the reduction in BDNF being released at the axon terminals of the granule neurons situated in the hilus., The BDNF acts upon the parvalbumin-expressing interneurons that are suggested to modulate neurogenesis. Additionally, knockout mice for the TrkB receptor of these parvalbumin-expressing cells showed the same phenotype. When administered an agonist of GABA(A) receptor, the BDNF knockout mice exhibited neurogenic levels similar to those observed in the wildtype. These results from Waterhouse et al. (2012) support the idea that the role of BDNF in hippocampal neurogenesis, may be mediated through parvalbumin-expressing interneurons located in the hilus that lays adjacent to the subgranular zone.

There have also been several studies that show an indirect relationship between BDNF and neurogenesis. For example, one study showed an increase in neurogenesis in rats that were subject to exercise and enriched environments (Bechara and Kelly, 2013); there were significantly increased levels of BDNF mRNA transcript in the hippocampi of exercised rats. Furthermore, knockout of the BDNF promoter in mice showed no difference in proliferation but reduced survival of progenitor cells; differentiation remained unaffected (Jha et al., 2011).

Jha et al. showed that environmental enrichment increased survival of progenitor cells but had no effect on differentiation. Additionally, another study showed that mice with heterozygous knockout of BDNF did not have show changes in proliferation compared to wildtype (Rossi et al., 2006); however, environmental enrichment increased both proliferation and neurogenesis in the wildtype while neither of these improvements was observed in the heterozygous knockout. Together these results support the idea that the effects of environmental enrichment and exercise on neurogenesis may be mediated by BDNF.

The aged hippocampus

The process of aging heavily affects the hippocampus along with learning and memory. One related physical change due to aging is decrease in hippocampal volume (Driscoll et al., 2003; Malykhin et al., 2008), which is accompanied by a decline of spatial memory and function (Rosenzweig and Barnes, 2003; Sykova et al., 2002). While no cell loss is observed, age-related degeneration of the hippocampus is characterized by reductions in dendritic spine densities, branching, and innervations (von Bohlen und Halbach, 2010). This degeneration of fibers affects the delicate circuitry that control communication via different neurotransmitters, including serotonin and acetylcholine, ultimately contributing to the volume reductions (von Bohlen und Halbach, 2010).

In non-human primates, a marked decline in BDNF expression in both soma and dendrites of neurons throughout the hippocampus has been observed with aging (Hayashi et al., 2001). The human hippocampus, however, maintains

a stable level of BDNF mRNA transcript while TrkB mRNA decreases with age (Webster et al., 2006). Furthermore, sustained BDNF expression is required to maintain spine density; a reduction of BDNF by knockout resulted in decreased spine density in adult cortical neurons (Vigers et al., 2012). Together, this data suggest that BDNF is required in maintaining certain physical properties of neurons, such as spine density.

Similarly, neurogenesis has also been found to decrease in primates with aging (Gould et al., 1999b). Along with studies on how neurogenesis affects hippocampus-dependent cognitive ability (Deng et al., 2009; Dupret et al., 2008), this decline may be implicated in age-related cognitive function. It has been found, however, that the overall population of neurons in the dentate gyrus of the aged hippocampus does not decrease (Calhoun et al., 1998; West, 1993). Thus, while decreased neurogenesis may be correlated with a decline in hippocampus-dependent learning and memory, it may be the rate of turnover and not necessarily the number of neurons in the dentate gyrus that underlie attenuated hippocampal function (von Bohlen und Halbach, 2010).

The aim of this study is to understand the effects of BDNF vector the aged primate entorhinal cortex on hippocampal neurogenesis. BDNF gene delivery in APP transgenic mice results in anterograde transport into the hippocampus from transduced neurons in the entorhinal cortex (Nagahara et al., 2009); this results in elevated BDNF at the outer molecular layer of the dentate gyrus and CA fields of hippocampus, the innervations sites of the layer II and III of. Interestingly, many of the subjects in the present study had unexpected alterations in BDNF

expression. In aged subjects, this altered pattern consists of elevated levels of BDNF in the granule cell layer with decreased levels in the hilus. Recent studies in our laboratory suggest that lower doses of BDNF vector (lower virus titer/few injection site) or perhaps shorter survival periods (1-6 month) can produce a pattern of BDNF expression reflecting anterograde transport of the BDNF from transduced cells in the entorhinal cortex. Nonetheless, the subjects in the present study showed an interesting pattern of BDNF expression in the hippocampus that can provide insight about the role of BDNF on neurogenesis in non-human primates.

MATERIALS AND METHODS

Animals

This study used the brain tissue of 27 adult male and female individuals of the Old World primate species Macaca mulatta, or more commonly known as the rhesus macaque. Monkeys were housed in individual cages at the California Regional Primate Research Center. Individuals were monitored closely, and were provided pain relief and antibiotics for any infections. There are two age groups: aged (18-26 years) and young adult (6-11 years). The experimental subjects received either adeno-associated viral (AAV) or lenti-viral vectors containing the BDNF gene injected into the entorhinal cortex, while control subjects received GFP vector or control solution injections. As shown in Table 1, aged subjects received AAV-BDNF (n = 8), lenti-BDNF (n = 3), lenti-GFP (n = 3), AAV-GFP (n = 2), or sham operation (n = 1). Young subjects received either AAV-BDNF (n = 6) or lenti-GFP (n = 4).

BDNF gene delivery & BrdU injection protocol

Subjects received AAV at a concentration of 1×10^{12} viral particles/ml or lentiviral vector at a concentration of 5×10^{8} infectious units/ml. Coordinates for stereotaxic guided injections into the entorhinal cortex were based on pre-surgery magnetic resonance imagery; 4 sites of injections were selected along the rostro-caudal extent of the entorhinal cortex on each side of the brain. Subjects were sedated intramuscularly with ketamine (25 mg/kg), and were then anesthetized with isofluorane. Afterward, the subject was placed in stereotaxic and the surface

of the skull was exposed. A craniotomy was performed to expose the brain region above the coordinates sites for the injection. At each site, a 50-µl Hamilton syringe with a 26-gauge needle was lowered to the coordinate and 20 µl of viral vector or control solution was infused into the entorhinal cortex at a rate of 1 µl per minute; following the infusion, there was a 2 minute wait period before the syringe was slowly raised out of the brain. BrdU (25mg/kg) was administered 3 weeks after BDNF vector delivery over 3 days. As summarized in Table 1, subjects survived between 1-24 months post-BrdU injection.

Immunohistochemistry

The subjects were either perfused transcardially with 4% paraformaldehyde (PFA) and 0.1% parabenzoquinone (PBQ) in 0.1M phosphate buffered saline, pH 7.4 (PBS) or saline. Brains from saline perfusions were postfixed in 4% PFA. Brains were removed, transferred to 30% sucrose solution, and then cryoprotected. Coronal sections were cut by microtome at a thickness of 40µm. The tissue was stored in either -20°C or -80°C freezers. Not all subjects had both hemispheres available for testing.

Three sets of immunohistochemistry were performed for this experiment. In each set, 3 sections were taken from each hemisphere. For the BrdU immunohistochemistry, tissue underwent incubation in 2N hydrochloric acid (HCl) for 1 hour to reveal the BrdU epitope. In the first set, the HCl was followed by blocking in 5% donkey serum and 0.25% Triton-X in tris-buffered saline for 1 hour. Afterward, they were incubated in primary antibody in blocking solution

over 1 night. BrdU was detected using mouse anti-BrdU (1:1500, Millipore MAB3222). In combination with BrdU, the following antibodies for phenotypic markers were used: rabbit polyclonal anti-Neuronal Nuclei (NeuN) (1:600, Biosensis R-3770-100) to detect mature neurons, goat polyclonal anti-doublecortin (DCX) (1:300, Santa Cruz Biotechnology sc-8066) to detect immature and migrating neurons. These sections were transferred to secondary antibodies, incubated for 2.5 hours, mounted, and left to dry under dark conditions at room temperature.

In the second set, in addition to the 2N HCl, Sudan Black was utilized to suppress autofluorescence. For this protocol, the tissue was incubated in 0.1M sodium borohydride ($NaBH_4$) in dH_2O followed by 0.5% Sudan Black in 70% ethanol for 30 minutes each. Because of the use of Sudan Black, permeabilization of the membranes was included in the hydrochloric acid as 0.5% Triton-X. Blocking was done using 5% donkey serum in tris-buffered saline without Triton-X. The tissue was left to incubate in primary antibody over 4 nights in blocking solution. BrdU was detected using mouse anti-BrdU (1:1500, Millipore MAB3222). In combination with BrdU, the following antibodies for phenotypic markers were used: rabbit polyclonal anti-Neuronal Nuclei (NeuN) (1:600, Biosensis R-3770-100) to detect mature neurons, goat polyclonal anti-glial fibrillary acidc protein (GFAP) (1: 300, Santa Cruz Biotechnology sc-6170) to detect mature astrocytes. The tissues were transferred to secondary antibody after 4 nights, incubated for 2.5 hours, mounted, and left to dry under dark conditions at 4°C.

In the third set, a heat retrieval protocol was used to further enhance fluorescent signal. Tissues were heated to 80°C then cooled in 0.01M Tris-HCl. They were then re-postfixed in 2% PFA/0.2% PBQ for 5 minutes. After this step, the protocol remained identical to that of the second set. For the antibodies, BrdU was detected using mouse anti-BrdU (1:1500, Millipore MAB3222). In combination with BrdU, the following antibodies for phenotypic markers were used: rabbit polyclonal anti-Neuronal Nuclei (NeuN) (1:600, Biosensis R-3770-100) to detect mature neurons, goat polyclonal anti-doublecortin (DCX) (1:300, Santa Cruz Biotechnology sc-8066) to detect immature and migrating neurons.

BrdU data was collected from all three sets of tissue. Doublecortin data was collected from only the third set due to poor labeling in the first. GFAP data was collected from the second set. NeuN data was collected from all three sets.

BDNF immunohistochemistry was performed light level with the use of diaminobenzidine (DAB). Tissue underwent heat-based antigen retrieval in 0.01M Tris-HCl then re-postfixed in 2% PFA/0.2% PBQ for 5 minutes. The tissue was then treated with 0.6% hydrogen peroxide in TBS. After blocking for non-specific binding in 5% donkey serum/0.25% Triton-X in TBS, the tissue was incubated in rabbit polyclonal anti-BDNF (1:2000, Chicago Proteintech 17465-1-AP) for 4 nights at 4°C. Afterward, the tissue was washed then incubated in biotinylated donkey anti-rabbit in blocking solution for 3 hours. This step was followed by incubation in avidin-biotin complex (ABC) for 2 hours. DAB was used as the last step to visualize BDNF immunoreactivity.

Groupings

The expression pattern of BDNF varied within the BDNF treatment group from a relatively normal pattern of BDNF expression (similar to control) to an altered pattern of BDNF expression. Therefore, Each hippocampus was categorized as one of three subgroups: control, BDNF treatment with unaltered expression, and BDNF treatment with altered expression (Table 2). Controls received no BDNF treatment, while subjects classified as having normal BDNF expression received treatment but the expression levels in the dentate gyrus are similar to that of controls. In both aged and young controls, BDNF immunoreactivity was highest in the hilus and CA3, with no or very little immunoreactivity in the granule cell layer. In aged animals that showed altered expression of BDNF, expression levels were significantly elevated in the granule cell layer, while decreased in the hilus and CA3. In young animals, altered expression showed intense BDNF immunoreactivity at the outer molecular layer and in the hilus, while the granule cell layer remained relatively unchanged.

Image analysis

Cells were quantified on every 24th section that included the hippocampus. Triple-labeling immunofluorescence was used to determine colocalization of different phenotypic markers. BrdU+ cells were quantified using confocal microscopy to confirm colabeling with other phenotypic markers. The Olympus Fluoview FV1000 was used with the program Olympus Fluoview ver. 1.7c. Each section was scanned along the dentate gyrus, and z-stacked

images were taken of cells at a step size of 0.5 μm. The number of cells was separated between SGZ and GCL.

BDNF immunoreactivity intensities were quantified using Image J. Images were taken on a SPOT camera (company) using an Olympic microscope (BX50) with 10x objective. BDNF immunolabeling was measured in each subregion of the hippocampus. Two sections were analyzed for each hippocampus. Two samples were taken for each subregion: granule cell layer, molecular layers (inner, middle, outer), hilus (adjacent to the subgranular zone). Quantification was based on the intensity of the image on a 0-255 scale of darkness.

Statistical analysis

To determine differences in cell counts, either one or two-way analysis of variance (ANOVA) was performed, followed by Student's *t* test. BDNF expression levels were analyzed by one-way ANOVA followed by post-hoc analysis using Student's *t* test. Mixed ANOVA was used for analyses using repeated data from individual subjects (e.g., branching vs. nonbranching). Differences were considered significant if $p < 0.05$. Figures express the data in means ± SEM. Young subjects that received BDNF vector into the entorhinal cortex were not included in statistical analyses due to low sample size ($n = 2$ per group).

In measuring the rate of neurogenesis, hippocampal sides that had no BrdU+ cells were excluded, resulting in a smaller sample size. Statistical analysis

was also performed on the combination of both aged BDNF treatment groups to provide additional insight.

RESULTS

BDNF expression in the hippocampus

BDNF immunoreactivity in different subregions of the dentate gyrus of different experimental groups are shown in Figure 1A-F. BDNF in the dentate gyrus in control subjects is expressed strongly in the polymorphic zone, hilus (Figure 1A, D), CA4, and CA3 (not shown). Additionally, there is almost no BDNF immunoreactivity in the GCL in control subjects. Although granule neurons express BDNF, the protein is trafficked through the axons, called mossy fibers, and is localized at the terminals located in the hilus and CA3. In the aged subjects that received BDNF treatment, there are two observed patterns of BDNF expression. The first pattern is similar to that of controls, with high levels of BDNF in the hilus and CA3 and very low levels in the GCL; however, some hippocampus in this group showed changes such as moderate elevation of BDNF in the GCL (Figure 1E). The other pattern of expression observed in aged treatment subjects show a different trend, with lower BDNF levels in the hilus and higher BDNF levels in the GCL (Figure 1F). The young BDNF group consisted of 4 subjects, with 2 exhibiting normal BDNF pattern of expression (Figure 1B) and the other 2 exhibiting elevation of BDNF expression in the inner molecular layer (Figure 1C). This pattern of BDNF expression is different from the changes observed in any of the aged subjects. Both altered patterns of expression of BDNF were unexpected based on rodent studies (Nagahara et al., 2009).

The BDNF expression levels in each region of the dentate gyrus were quantified (Figure 2). As indicated in the method sections, the young BDNF

subjects were not included in the statistical analyses due to only 2 subjects per group. Repeated mixed ANOVA revealed a significant interaction effect of groups and region ($F = 4.17$, $p < 0.0150$). Subsequent ANOVA and post-hoc analyses revealed that the aged subjects with altered BDNF expression had reduced levels in the hilus ($F = 13.29$, $p < 0.0001$) compared to aged controls ($p < 0.0001$) and aged BDNF subjects with normal expression ($p < 0.0004$). No significant difference was found between the aged controls and the aged BDNF subjects with normal expression. Furthermore, the aged subjects with altered BDNF expression showed elevated levels in the GCL ($F = 71.43$, $p < 0.0001$) compared to aged controls ($p < 0.0001$) and aged normal BDNF ($p < 0.0001$). There was no difference in the pattern of BDNF expression between aged and young control subjects.

Immunohistochemistry

Cells were characterized using confocal microscopy and were identified either in the GCL or the SGZ (Figure 3A, B). BrdU+ cells are particulate inside the nucleus and are approximately the same size as NeuN+ cells (Figure 3C, F). DCX+ cells are cytoskeletal (Figure 3G) and encase BrdU/NeuN (Figure 3F). DCX+ cells may show branching (Figure 3E), which was used as a measure of maturation in neurons.

BrdU+ cells in the dentate gyrus

The number of BrdU+ cells is used as a measure of surviving cells that proliferated during the 3-day BrdU injections 3 weeks post-BDNF gene delivery. There is a reduction in the number of BrdU+ cells in the aged control group compared to the young control group ($p < 0.02$). The aged BDNF subjects showed an increase in BrdU+ cells compared to aged controls with a 497% increase in unaltered and 917% increase in altered expression, but these effects were not significant (unaltered: p=0.35; altered: $p = 0.09$; Figure 4A). One-way ANOVA (excluding young BDNF groups), however, showed no statistical significance across the aged groups and young controls ($F = 1.69$, $p < 0.187$). Additional analyses combining the aged BDNF groups did not reach significance ($F = 1.97$, $p = 0.16$). However, the trend of increased BrdU+ cells in the aged treatment group compared to the controls is still observed ($p = 0.11$) (Figure 4B).

BrdU+ cells expressing either DCX or NeuN

The amount of neurogenesis was quantified as the number of BrdU+ cells that also expressed at least either DCX or NeuN. These cells were classified as neuronal BrdU+ cells. The amount of neuronal BrdU+ cells provides a measure of how many cells proliferated at the time of BrdU injection, differentiated into neurons, and survived until the time of sacrifice. A one-way ANOVA (excluding young BDNF groups) across the aged groups and young control revealed differences between groups ($F = 11.84$, $p < 0.0001$). Post-hoc analysis using Student's t test revealed differences between young controls and aged controls (p

< 0.006) (Figure 5A). This age effect showed 8-fold higher BrdU+ cells in the young controls compared to aged controls. No significance was found between the aged controls and either BDNF treatment groups. In the young BDNF altered group, there were higher numbers of total BrdU+ cells, but the number of neuronal BrdU+ cells was similar to those of the control. Analyses using the combined BDNF treatment groups (Figure 5B) resulted in similar significant effects ($F = 18.21$, $p < 0.0001$; young control vs. aged control $p < 0.0001$).

Rate of neurogenesis in the dentate gyrus

While aged subjects with BDNF treatment showed higher levels of BrdU+ cells, only a low percentage of these newborn cells differentiated into neurons. Thus, another parameter at looking neurogenesis would be assessing the rate as measured by the percentage of BrdU+ cells that express a neuronal marker. One-way ANOVA revealed differences among the aged subjects and young controls ($F = 4.45$, $p < 0.02$). Post-hoc analyses using Student's t test revealed a strong trend for a decrease rate of neurogenesis in aged subjects with altered expression compared to aged controls ($p = 0.06$). A similar effect was found when comparing aged controls to aged treatment with unaltered expression ($p = 0.08$) (Figure 6A). This trend shows over 90% reduction in the rate of neurogenesis in aged BDNF treatment subjects vs. control. The rate of neurogenesis does not change with age as revealed by the comparison between young and aged controls. While no conclusion can be made of the young treatment groups, there is a similar trend with decreasing rate of neurogenesis with BDNF treatment. Combining the

two aged BDNF groups showed a significant difference between aged subjects and all aged BDNF subjects ((ANOVA $F = 6.92$, $p < 0.004$), post-hoc $p < 0.01$) (Figure 6B).

Total number of DCX+ cells as another measure of neurogenesis

DCX is expressed in the adult macaque hippocampus for a period of about 6 months. Thus, the number of immature neurons at the time of death is can be used as a measure of neurogenesis that occurred over the last 6 months. One-way ANOVA showed a significant difference between groups ($F = 10.36$, $p < 0.0001$) (Figure 7A). Post-hoc analyses revealed a significant reduction in the number of DCX+ cells in aged controls versus young controls ($p < 0.01$); similar reductions were also observed in aged BDNF groups compared to young controls ($p < 0.01$). Comparisons between aged treatment groups and aged controls were not significantly different. Combining the two aged BDNF groups (ANOVA $F = 15.98$, $p < 0.0001$) revealed a trend of reduced DCX+ cells in BDNF subjects compared to controls ($p = 0.13$; Figure 7B).

Localization of DCX+ cells to either the SGZ or GCL

BDNF is known to have an effect on neuronal growth and maturation in development. As such, one measurement of neuronal maturation in the adult hippocampus is localization of the DCX+ cells. Neurons are born in the SGZ and migrate into the GCL as they mature (Figure 8). Therefore, the effects of BDNF and aging on the location of DCX+ cells were examined. Repeated mixed

ANOVA (subject group x locations) revealed no interaction effect of subject group and location of DCX+ cells ($F = 0.635$, $p = 0.60$) (Figure 9).

Branching of DCX+ cells as another measure of maturation

The formation of processes is also used as a measure of neuronal maturation (Figure 8). Because DCX is localized to the cytoskeleton, it is possible to quantify the number of cells with processes. Repeated mixed ANOVA showed a significant interaction effect of group and branching of DCX+ cells ($F = 8.06$, $p < 0.0004$). Subsequent ANOVA revealed a significant difference between the aged groups and young controls ($F = 9.98$, $p < 0.0001$). Post-hoc analyses revealed that there is a significant increase in the number of branching DCX+ cells in young controls compared to aged controls ($p < 0.0002$). Aged controls showed no difference compared to either aged BDNF treatment group in the branching of cells. One-way ANOVA for non-branching cells did not reveal any significant differences between aged groups and young controls. While no conclusions can be made with the young BDNF subjects, it was observed that in subjects with both normal and altered expression of BDNF, there is a greater amount of DCX+ cells with no branching compared to the control (Figure 10).

BrdU+ cells do not colocalize with GFAP

GFAP in this study was used as a marker for mature astroctyes (Figure 11). Quantification of cells colabeled with BrdU and another phenotypic marker

revealed that cells are either neuronal or are unidentified. Confocal microscopy was used to determine colabeling of BrdU and GFAP across 3 sections from each subject. There were a total of 401 BrdU+ cells in the GCL and SGZ identified in this test. No BrdU+ cell was colabeled with GFAP. Because there were no co-labeled cells, no statistical analyses were performed.

DISCUSSION

BDNF expression in the dentate gyrus

This project examined the effects of BDNF gene delivery into the entorhinal cortex on neurogenesis in the subgranular zone in the hippocampus. In the present study, both young and aged controls showed a pattern of BNDF that exhibited high levels in the hilus and CA3 with very low expression in the granule cell layer, reflecting BDNF localization at axon terminals in the hilus (Conner et al., 1997). In contrast, aged subjects that received BDNF gene delivery to the entorhinal cortex showed different patterns of BDNF expression in the hippocampus, ranging from relatively normal to altered expression patterns. In general, hippocampus with relatively normal patterns of BDNF expression had limited BDNF gene delivery to the ipsilateral entorhinal cortex as indicated by BDNF immunoreactivity. In contrast, the altered pattern of BDNF expression in the hippocampus showed high levels of BDNF immunoreactivty in the ipsilateral entorhinal cortex. Two of the young subjects that received BDNF vector displayed high levels of BDNF labeling in the inner molecular layer; this might reflect transduction of mossy cells in the hilus that innervated this region (Frotscher et al., 1994). The other two young BDNF treatment subjects showed a pattern of BDNF expression that represents the pattern observed in controls.

The altered expression in aged subjects was unexpected as a previous study showed that gene delivery into the entorhinal cortex resulted in anterograde transport of BDNF into the hippocampus, primarily to the outer molecular layers of the dentate gyrus and CA fields of transgenic mice (Nagahara et al., 2009).

Furthermore, recent studies in our laboratory found that MRI-guided delivery of BDNF vector into the entorhinal cortex of rhesus monkeys, which increases target accuracy, results in BDNF labeling in the outer molecular layer of the dentate gyrus; the survival period of these subjects is generally 1 month for the young subjects (n = 8) and 6 months of the aged subject (n = 2). These treatments included lower titer as well as fewer injection sites. These treatments resulted in expression restricted to anterograde transport of BDNF to the outer two-thirds of the molecular layer and lacunosum-moleculare layers of the CA fields in both young and aged subjects. Similar injections with GFP gene delivery to the entorhinal cortex displayed intense GFP labeling at the outer molecular layer that is reflective of anterograde transport from the entorhinal cortex. It is not clear why we did not observe this pattern in our aged subjects, but the higher dosages of gene delivery and/or the longer period of survival may have played a role in the altered expression of BDNF. This altered expression of BDNF in aged subjects may reflect the retention of BDNF to granule neurons instead of being trafficked to axon terminals located in the hilus and CA3 (Wetmore et al., 1994). This would explain the decrease of BDNF levels in the hilus and CA3 accompanied by an increase in the GCL in these subjects with altered expression.

Age-related decline in the survival of adult-born neurons in the dentate gyrus

Previous studies have shown that both proliferation and survival of new neurons decrease with age in both rodents (Heine et al., 2004; Kuhn et al., 1996; McDonald and Wojtowicz, 2005; Rao et al., 2006) and primates (Aizawa et al.,

2011; Aizawa et al., 2009; Gould et al., 1999b). Similar to previous findings, aged subjects in this study exhibit significantly reduced proliferation compared to young controls. Additionally, the aged controls in this experiment exhibited significantly fewer newborn neurons, as measured by BrdU+ cells colabeled with either NeuN or DCX, compared to young controls. This decrease in total amounts of newborn neurons may reflect a smaller population of neural progenitor cells (NPC). The population of NPCs has been found to decrease with aging in both primates (Aizawa et al., 2011) and rodents (Rao et al., 2006). Comparisons on the percentage of the BrdU+ cells that colabeled with a neuronal marker revealed that there is no difference between young and aged controls, which has been reported (Leuner et al., 2007; McDonald and Wojtowicz, 2005; Rao et al., 2006). In contrast to the previous studies, however, the rate of neurogenesis has also been shown to decrease with aging (Heine et al., 2004).

Additionally, the overall number doublecortin-expressing cells may be another measure of neurogenesis. Doublecortin expression in newborn granule neurons is expressed for around 6 months in primates (Kohler et al., 2011). The present study found that aged subjects had reduced neurogenesis within the last 6 months of survival compared to young subjects as measured by total number of doublecortin-expressing cells. These findings are consistent with previous studies that show decreased neurogenesis with aging (Aizawa et al., 2009; Gould et al., 1999b).

BDNF gene delivery results in a strong trend of increased cell proliferation but does not affect cell fate

BDNF has been reported to have effects on cell proliferation in the adult hippocampus. Reduction of BDNF levels by knockout in mice leads to increased cell proliferation in the subgranular zone (Waterhouse et al., 2012). Overall, the mice with reduced hilar BDNF level showed a decrease in the rate of neurogenesis since proliferation increased while the number of cells that differentiated did not change. Interestingly, the aged BDNF treatment groups exhibited a trend of increased cell proliferation. This trend showed an increase of 917% in altered expression and 497% in unaltered expression compared to controls. However, in the present study, there was no difference in the number of BrdU+ cells exhibiting a neuronal phenotype between control and treatment groups. This indicates that the rate of neurogenesis is decreased with BDNF gene delivery to the entorhinal cortex. This trend was observed in both aged treatment groups that showed altered and unaltered expression of BDNF. The aged treatment group that exhibited unaltered expression of BDNF still had minor alterations; the levels of BDNF in the GCL was still significantly higher than in controls, along with slightly decreased BDNF in the hilus. These effects may reflect the smaller degree of proliferation in these subjects. These findings are consistent with studies observing BDNF depletion in rodent models (Chan et al., 2008; Lee et al., 2002).

BDNF is also implicated in having effects on survival of newborn cells in the adult hippocampus. Knockout of BDNF in mice results in decreased survival

of newborn neurons (Choi et al., 2009; Sairanen et al., 2005). The study conducted by Sairanen et al. showed that the number of BrdU+ cells diminished 3 weeks after post-BrdU injection compared to the amount of cells 24 hours post-injection in BDNF knockout mice, while no decrease was found in control mice; this finding suggests that BDNF plays a role in the survival of these newly proliferated cells. BDNF levels were decreased in the hilus of these aged subjects; based on the previous studies, this might be expected to result in decreased survival of newborn cells. Similar to previous findings, the current study found that reduced levels of BDNF in the hilus led to an overall decreasing trend in the number of BrdU+ cells with neuronal phenotype. Both aged treatment groups exhibited only about 30% of the number of newborn neurons that was present in controls. This may be due to decreased survival of the newborn cells as a result of BDNF reduction in the hilus.

Neuronal differentiation of newborn cells is also affected by BDNF. Reduction of BDNF levels in the hippocampus has been shown to lead to increased proliferation while the number of BrdU+ cells that differentiate into neurons remains the same (Chan et al., 2008; Lee et al., 2002; Waterhouse et al., 2012); this results in an overall decrease in the percentage of BrdU+ cells that become neurons. Furthermore, infusion of BDNF into the rat hippocampus results in increased proliferation along with differentiation into neurons (Scharfman et al., 2005). Based on the literature, a decrease in BDNF in the hippocampus leads to decreased neuronal differentiation while increased BDNF leads to increased neuronal differentiation. Together, these results suggest that

BDNF may play a part in basal levels of differentiation in the adult hippocampus. In the current study, there was an observed decrease in the rate of neuronal differentiation as measured by the number of BrdU+ cells that colabeled with NeuN or DCX of aged subjects that received BDNF treatment. This is consistent with the findings of previous studies in which BDNF reduction in the hippocampus resulted in decreased neuronal differentiation.

The mechanism by which BDNF modulates neurogenesis in the adult hippocampus may be mediated through parvalbumin-expressing GABAergic interneurons located in the hilus (Waterhouse et al., 2012). Knockout of the BDNF gene reduces the amount of BDNF produced by the granule neurons, resultingin a decrease in BDNF in the hilus, a pattern that was observed in aged subjects with altered BDNF expression. The same study found that the same BDNF knockout rats exhibited a reduction in glutamic acid decarboxylase 65 (GAD 65), an enzyme that synthesizes gamma-aminobutyric acid (GABA). GABA works as an inhibitory neurotransmitter in the nervous system. The deficits in neurogenesis were restored after administration of phenobarbital, an agonist of the GABA(A) receptor. This suggests an effect of BDNF that is mediated through GABAergic cells in the hilus. Additionally, similar defects in neurogenesis were observed when TrkB was selectively removed from these GABAergic cells by Cre-recombination. Waterhouse et al. suggested that these GABAergic cells are parvalbumin-expressing interneurons present in the hilus, which lays adjacent to the subgranular zone. These GABAergic interneurons have been found to be located near type 2 progenitor cells which contain

GABA(A) receptors (Tozuka et al., 2005). Together, these results suggest that BDNF released by granule neurons act upon GABAergic cells which in turn produce GABA that stimulate type 2 progenitor cells. Furthermore, the use of GABA(A) receptor agonists has induced neuronal differentiation while decreasing proliferation in adult hippocampal progenitor cells, further providing evidence for GABA as having a role in hippocampal neurogenesis (Seri et al., 2001; Tozuka et al., 2005). In our aged subjects with altered BDNF expression, BDNF seems to be retained within the cell bodies of the granule neurons rather than being trafficked out into the mossy fibers and subsequently to the axon terminals in the hilus and CA3. Thus, this retention of BDNF to the granule neurons, and subsequently decreased levels of BDNF in the hilus, could have resulted in decreased activation of GABA cells. This potential mechanism explains the trend for increased cell proliferation with decreased rates of neuronal differentiation observed in the current study.

Maturation of newly born granule cells

Since doublecortin is a marker of immature neurons, it is possible to track the stage of maturation by examining different characteristics of DCX+ cells, such as the location and the amount of branching. Therefore, in this study, DCX+ cells were classified as either having branching or not and as either being located in the SGZ or GCL.

The first measure of neuronal maturation is comparing the relative numbers of doublecortin-expressing cells that were localized to either the SGZ or

GCL. DCX+ cells migrate from the SGZ into the GCL as they mature (Kempermann et al., 2003). Newborn neurons originate from the SGZ and migrate into the GCL. Migration of cerebellar granule neurons during development is stimulated by BDNF (Borghesani et al., 2002). One study using GFP-encoding retrovirus to visualized newly generated cells found that depletion of BDNF in the hippocampus results in an increased number of GFP/NeuN+ cells in the SGZ compared to controls (Chan et al., 2008). In the present study, aged subjects with BDNF treatment showed only about 30% of the number of DCX+ cells in the SGZ observed in controls; this trend was not found with DCX+ cells in the GCL. DCX+ cells in the SGZ are newly proliferated and depletion of BDNF results in reduced survival of newborn neurons (Choi et al., 2009). It may be possible that the decrease in hilar BDNF observed in these aged subjects result in decreased numbers of newly born DCX+ cells in the SGZ. The BDNF treatment group with relatively unaltered expression of BDNF still showed a slight decrease in the hilus, which may account for the reduced number of DCX+ cells in the SGZ.

The second measure of maturation involved the branching of doublecortin-expressing cells. DCX+ cells that lack branches are attributed to being in the proliferative stage and represent newborn neurons, while cells that have extended processes are more mature (Plumpe et al., 2006). In the postnatal rat hippocampus, BDNF has been reported increase the extension of dendrites of cultured nonpyramidal neurons (Marty et al., 1996). This BDNF effect of enhanced arborization was also observed in cultures of neurons of other brain

regions, including the cortex (Horch and Katz, 2002) and neocortex (Jin et al., 2003). Furthermore, BDNF depletion in postnatal mice by Cre-recombination resulted in a decrease in dendritic complexity and arborization of cortical neurons (Gorski et al., 2003). In murine cortical neurons, sustained BDNF expression is necessary for maintenance of dendrites; BDNF depletion by knockout resulted in decreased spine density (Vigers et al., 2012). In the current study, aged subjects that received BDNF vector showed a trend for decreasing nonbranching DCX+ cells; both groups exhibited only about 30% of the number of cells present in controls. This may be due to the decrease in hilar BDNF exhibited by the aged subjects that received BDNF gene delivery. Decreased BDNF in the hilus has been shown to decrease survival of newborn neurons. Since nonbranching DCX+ cells represent newborn neurons, decreased BDNF in the hilus may explain this trend. Even in the unaltered group, there is a decrease in hilar BDNF that could explain the decreased survival of newborn cells. Interestingly, the young subjects with altered BDNF expression exhibited a 700% increase in the number of nonbranching DCX+ cells compared to controls; this trend may be explained by the effect of BDNF on newborn neurons. DCX+ cells that exhibit no branching are representative of newly proliferated neurons (Plumpe et al., 2006). BDNF has been shown to increase survival of newborn neurons by decreasing apoptosis during development (Bhave et al., 1999). The elevated levels of BDNF in the inner molecular layer displayed by these young subjects may have an influence on the survival of newborn neurons.

Potential fates of BrdU+ cells

In the current study, about a third of the BrdU+ cells were colabeled with a neuronal marker in aged controls, while aged subjects receiving BDNF vector showed significantly lower amounts. This leaves a very large population of unidentified BrdU+ cells. Although the amount of newborn cells decrease with age, the fates of these cells have mostly (over 80%) been reported as neuronal in both primates (Gould et al., 1999b; Kohler et al., 2011) and rodents (McDonald and Wojtowicz, 2005; Seri et al., 2001). The rate of neurogenesis has been shown to remain consistent with aging (Leuner et al., 2007).

GFAP was used to determine whether these unidentified BrdU+ cells that become astrocytes. Based on previous studies in primates, the number of newly born cells that become astrocytes is very low, ranging from none (Yagita et al., 2001) to about 5% of all BrdU+ cells (Bruel-Jungerman et al., 2005). In the present study, none of the newborn cells colabeled with GFAP, a marker for astroctyes. In postnatal rodents, full expression of GFAP in hippocampal astrocytes requires around 20 days (Pixley and de Vellis, 1984), although it has been suggested that GFAP expression is dependent on function rather than time (Missler et al., 1994). GFAP expression in newborn hippocampal cells in adult macaques has been found to be as early as 2 weeks post-BrdU injection (Gould et al., 2001). All of the subjects in the current study survived at least one month post-BrdU injection, thus the absence of BrdU/GFAP+ cells suggest that these newborn cells are not astrocytes. The very low percentage of cells that do become

astrocytes as reported in previous studies may explain the lack of observed BrdU/GFAP+ cells.

Based on the absence of GFAP/BrdU+ cells across all subjects, there still remains an overwhelming population of unidentified BrdU+ cells. There are several explanations of unidentified BrdU+ cells. Although not tested for in this experiment, microglia might explain the unidentified BrdU+ cells. One studied found that stereotaxic insertions of needles into the hippocampus led to microglial invasions (36% of all BrdU+ cells) and other unidentified non-neuronal BrdU+ cells (Song et al., 2013). The subjects in this study underwent stereotaxic insertions that involved crossing the hippocampus, which may explain the elevated amounts of unidentified cells. While these cells may colocalize with untested markers, it has also been suggested that these newborn cells remain undifferentiated until a later time when function is determined (Kempermann, 2002; Prickaerts et al., 2004). Although up to 20% of BrdU+ cells have been previously reported as unidentified (Cameron et al., 1993; Czeh et al., 2002; Kempermann and Gage, 2002), it was not to the same extent as observed in the present study. This could be due to differences in strictness of criteria, immunolabeling, and tissue quality. Whether these BrdU+ cells are differentiated with no known marker or are undifferentiated is yet to be determined.

Summary

The findings of this study reveal that neurogenesis is reduced with age in the non-human primate, as measured by the number of BrdU+ cells that adopt a

neuronal phenotype and the number of DCX+ cells. These findings are consistent with previous reports (Aizawa et al., 2011; Gould et al., 1999b). Furthermore, the rate of neuronal differentiation, as measured by the percentage of BrdU+ cells expressing either NeuN or DCX, does not change with aging; this finding is also consistent with previous studies (Leuner et al., 2007; McDonald and Wojtowicz, 2005; Rao et al., 2006). Notably, BDNF gene delivery to the entorhinal cortex of aged primates increased the number of newly born hippocampal cells, yet surprisingly worsening the proportion of cells adopting a neuronal fate. A possible mechanism underlying this reduction in the rate of neuronal differentiation is the observation that BDNF delivery in aged monkeys actually reduced the level of hilar BDNF. This is consistent with previous reports that BDNF reduction in the hippocampus in rodents leads to a decrease in the rate of neuronal differentiation as a result of increased cell proliferation without promoting neuronal differentiation (Waterhouse et al., 2012; Chan et al., 2008). Our findings suggest that BDNF in the hippocampus plays an important role in adult neurogenesis.

Future directions: BDNF and Neurogenesis

While this study examined the effects of BDNF gene delivery into the entorhinal cortex on hippocampal neurogenesis, whether BDNF actually increases neurogenesis is unclear. The aged subjects that received BDNF vector showed a decline in BDNF expression in the hilus, and showed a trend for increased proliferation with significantly lower rates of neuronal differentiation. The

current study did not examine the effects of increased hilar BDNF levels on neurogenesis. Additionally, this study used mainly aged subjects that received BDNF gene delivery. Future experiments could include a larger sample size of younger animals for a better understanding of the aging effects on neurogenesis. Furthermore, there are still a high percentage of unidentified BrdU+ cells in this study. This may be due to the limited markers that were tested; there are many markers that can still be tested for by immunohistochemistry to identify the properties of these BrdU+ cells. Next, the pathway and mechanism by which BDNF affects neurogenesis is unclear. So far, it has only been suggested that reduction of BDNF decreases activation of GABAergic interneurons. As a result, there is a reduction in GABA which stimulates GABA(A) receptors present on type 2 progenitor cells. Other types of cells and molecules may play a role in this process; immunohistochemistry and rodent knockout models could be used to determine the molecules involved in and required for adult neurogenesis. Elucidating this mechanism will provide insight on the key factors that play a role in neurogenesis.

Understanding the effects of BDNF on neurogenesis and its mechanism will provide insight on using BDNF as a therapeutic approach in treating hippocampus-dependent cognitive decline. BDNF gene delivery into the entorhinal cortex has already been shown to ameliorate cognitive decline in mice and primate models of AD. Additionally, BDNF infusion into the rat hippocampus has also shown similar effects. Furthermore, hippocampal neurogenesis has also been shown to improve cognitive ability in rodents. Thus,

the effects of BDNF on neurogenesis are of importance in improving cognitive decline in animal models. This would have implications on the clinical treatment of cognitive decline, such as in aging and Alzheimer's disease.

TABLES AND FIGURES

Table 1: Summary of individuals.

Subject	Age	Gender	Perfusion	Treatment	Survival (days)
17176	28	f	PBQ	Lenti-BDNF	138
18671	25	f	PBQ	Lenti-BDNF	139
19660	24	m	PBQ	Lenti-BDNF	139
H 21011	24	m	PBQ	AAV-BDNF	25
21684	24	m	Saline	AAV-BDNF	697
22241	24	m	Saline	AAV-BDNF	685
22295	26	f	Saline	AAV-BDNF	666
23762	24	m	Saline	AAV-BDNF	865
24647	22	f	Saline	AAV-BDNF	664
25722	18	f	PFA	AAV-BDNF	31
26169	18	f	None	AAV-BDNF	23
20348	26	m	Saline	Lenti-GFP	702
22450	23	m	PBQ	Lenti-GFP	132
24453	23	m	PBQ	Lenti-GFP	153
22302	23	m	Saline	AAV-GFP	690
22420	23	m	Saline	AAV-GFP	716
22612	25	m	Saline	Vehicle	634
31581	11	f	Saline	AAV-BDNF	356

Table 1: Summary of individuals, continued.

31703	11	f	Saline	AAV-BDNF	351
35443	6	m	Saline	AAV-BDNF	384
35600	6	m	Saline	AAV-BDNF	384
28923	10	f	PBQ	AAV-BDNF	141
30279	8	f	PBQ	AAV-BDNF	119
31545	6	m	PBQ	Lenti-GFP	293
32812	12	m	PBQ	Lenti-GFP	138

Table 2: Summary of hippocampal sides

	Control	Treatment Unaltered Expression	Treatment Altered Expression
Aged	9	8	12
Young	8	2	2

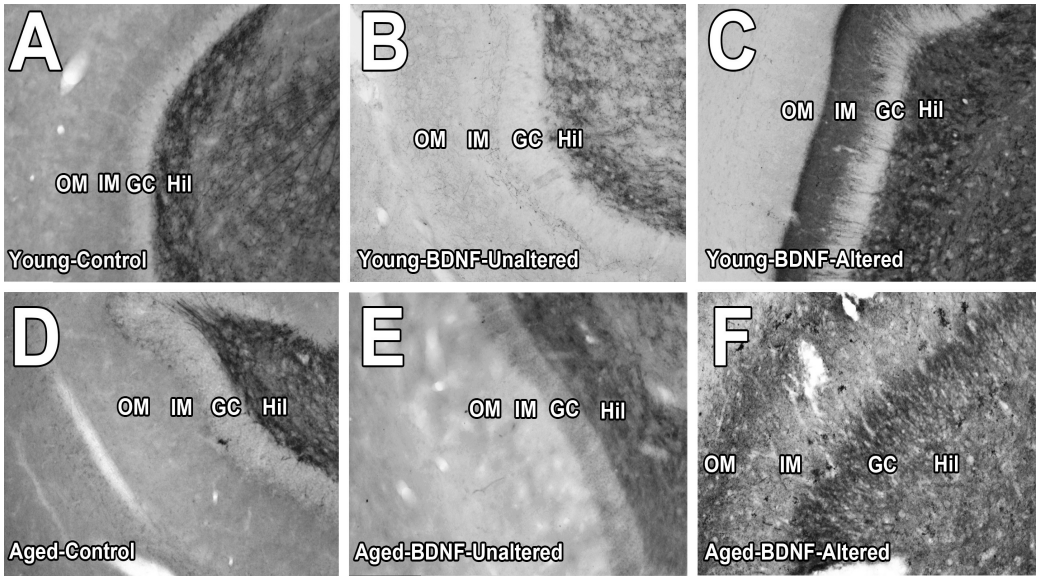

Figure 1: BDNF expression in the dentate gyrus. BDNF immunoreactivity in young and aged control subjects is most intense in the hilus (Hil) with very low expression in the granule cell layer (GC) (Fig. 1A, D). Some hippocampishowed relatively normal pattern of expression in both young and aged subjects following BDNF gene delivery into the entorhinal cortex (Fig 1B, E) though some minor changes were observed like elevated BDNF expression in the GCL (Fig. 1E). Some young animals with BDNF treatment displayed increased BDNF immunoreactivity in the inner molecular layer (IM), but showed no changes in the GCL or hilus (Fig. 1C). In contrast, some hippocampi in BDNF-treated aged subjects showed altered patterns of BDNF expression with an elevation in the GCL and a decrease in the hilus. In these subjects, the intensity levels of the BDNF immunoreactivity in the GCL are higher than in the hilus (Fig. 1F).

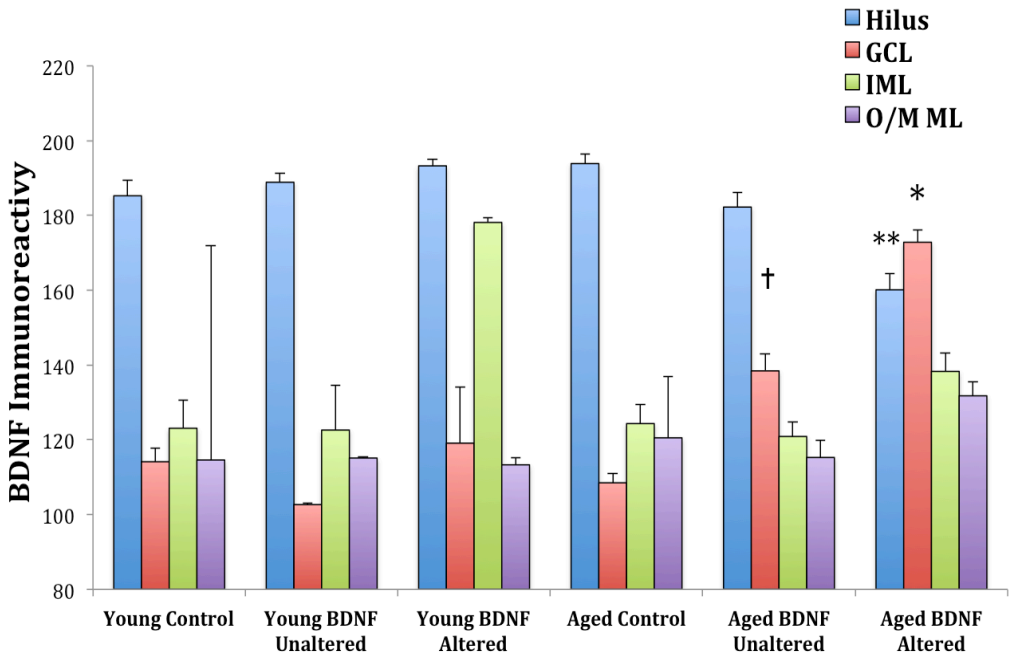

Figure 2: Quantification of the BDNF immunoreactivity in several locations of the dentate gyrus. It was observed that aged subjects with BDNF treatment and altered expression showed very high levels of BDNF in the GCL (*$p < 0.001$) and decreased levels in the hilus (**$p < 0.001$) compared to controls. Aged subjects with relatively unaltered expression showed a significant increase in the GCL compared to controls (†$p < 0.001$). Unlike the subjects with altered expression, this other group of aged BDNF treatment subjects did not show a significant reduction of BDNF levels in the hilus ($p = 0.089$). In comparison, in the young subjects, neither of these changes was observed. Instead, there was a trend of increasing BDNF in the IML compared to other young subjects. This figure also displays an average of BDNF expression between the outer and middle molecular layers. There were no significant differences in these two areas.

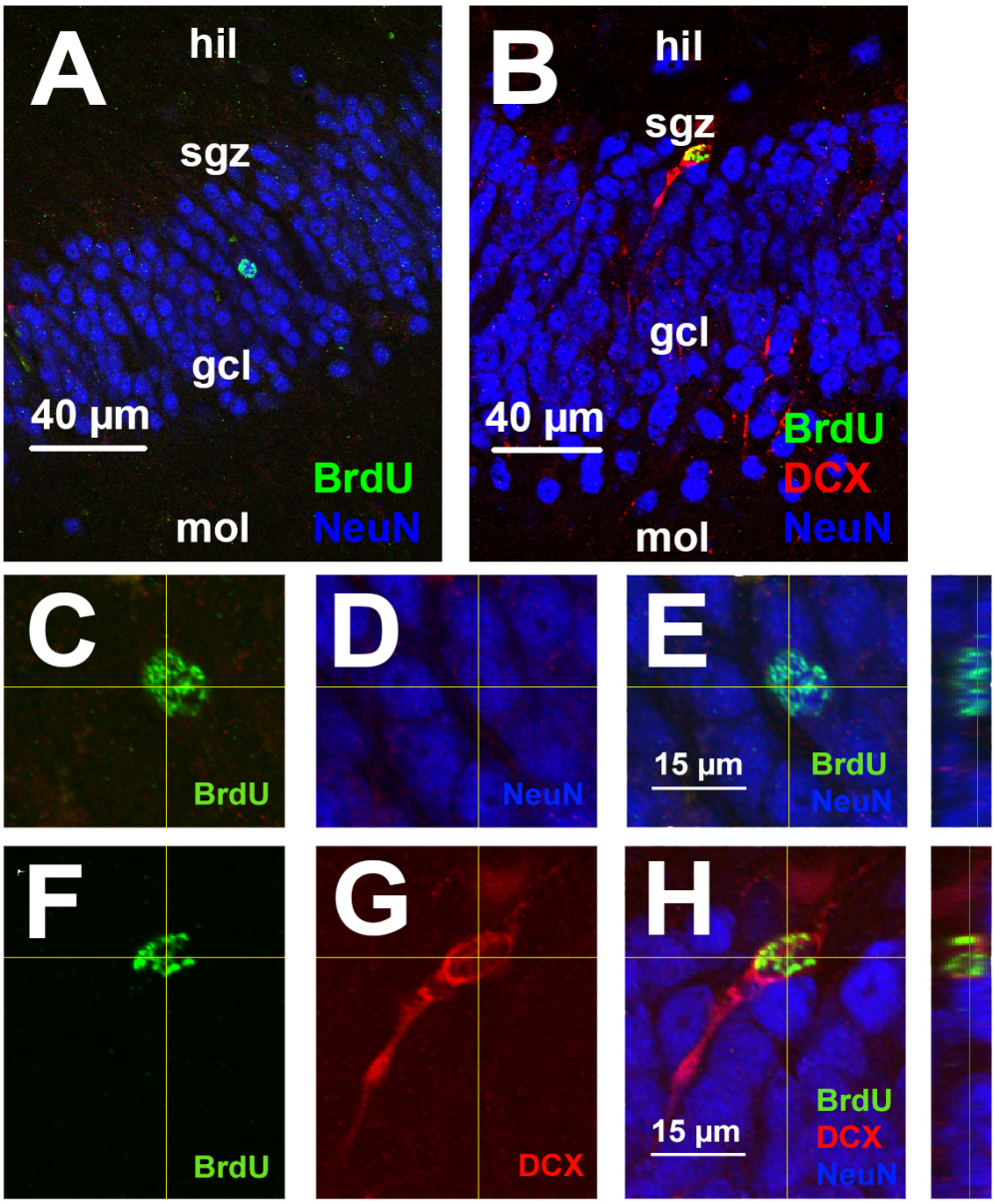

Figure 3: BrdU+ cells expressing a neuronal marker. Shown are two cells that are colabeled with BrdU and either NeuN or DCX. The BrdU localizes in the nucleus with NeuN (Fig. 3E), while the DCX is localized to the cytoskeleton around the cell (Fig. 3H). Also are the relatively locations of each cell. BrdU+ cells that colabel with NeuN are usually localized in the GCL; newborn in neurons in the SGZ mature and migrate into the GCL. This reflects cells that were currently migrating or not yet fully matured. Figure 3A/B provides the original image taken with of the cell shown in 3C-E/F-H (zoomed in 4x) indicating the cell's position relative to each subregion of the dentate gyrus (hil, hilus; sgz, subgranular zone; gcl, granule cell layer; mol, molecular layer).

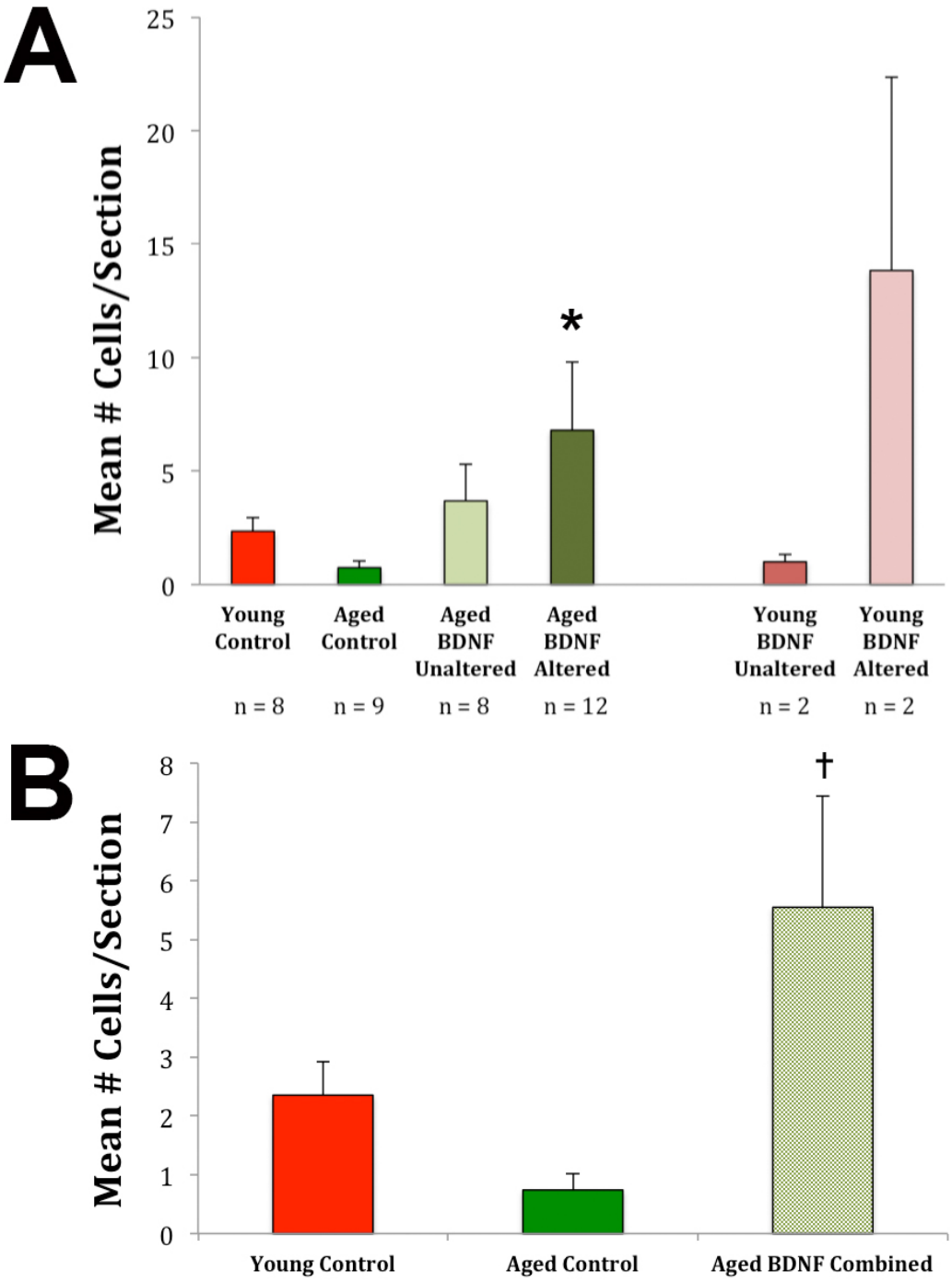

Figure 4: Number of BrdU+ cells in the SGZ and GCL.

Figure 4: Number of BrdU+ cells in the SGZ and GCL, continued.
(**A**) The number of BrdU+ cells does not differ significantly across the treatment groups in aged subjects (ANOVA $F = 1.69$, $p = 0.19$). However, the aged control showed a 69% reduction in the total number of BrdU+ cells compared to the young control group; statistical analysis of only these two groups showed a significant change (*$p < 0.02$). Compared to the aged control, both aged BDNF groups showed an increase in total BrdU+ cells with a 497% increase in the aged BDNF unaltered group and a 917% increase in the aged BDNF altered groups. A statistical comparison between each aged BDNF groups controls with the aged control reveal a non-significant change in both the aged BDNF unaltered group ($p = 0.35$) and the aged BDNF altered ($p = 0.09$). Interestingly, the young BDNF Altered group (N=2) showed a 600% increase compared young control. (**B**) An additional statistical analysis was performed with the aged BDNF groups combined, but this did not reach significance ($F = 1.97$, $p = 0.16$); however, there is a strong trend of increased number of BrdU+ cells in aged subjects with BDNF treatment compared to aged controls (†$p = 0.11$).

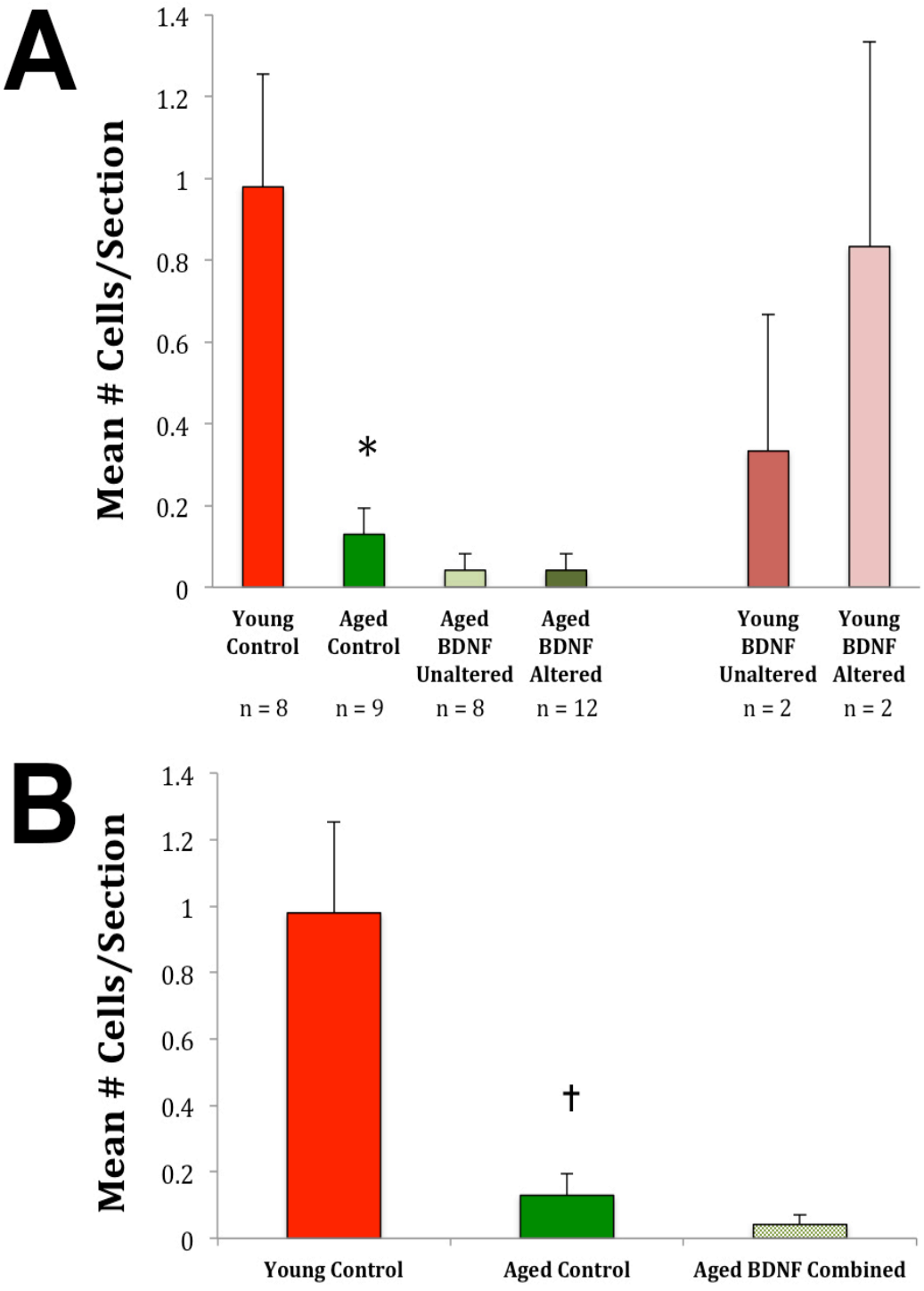

Figure 5: Number of BrdU+ cells expressing a neuronal marker.

Figure 5: Number of BrdU+ cells expressing a neuronal marker, continued. (**A**) One-way ANOVA revealed a difference among the control groups and the aged BDNF groups ($F = 11.78$, $p < 0.0001$). Neurogenesis is decreased in aged controls compared to young controls (*$p < 0.006$), but no statistical significance was found between the aged controls and either aged BDNF unaltered ($p = 0.25$) or aged BDNF altered ($p = 0.30$). (**B**) One-way ANOVA revealed a difference among the three groups ($F = 18.21$, $p < 0.0001$). The number of BrdU+ cells adopting a neuronal fate is significantly reduced in aged controls compared to young controls (†$p < 0.0001$). There is no observed significant difference between the aged controls and the combined group of BDNF treatment subjects ($p = 0.17$).

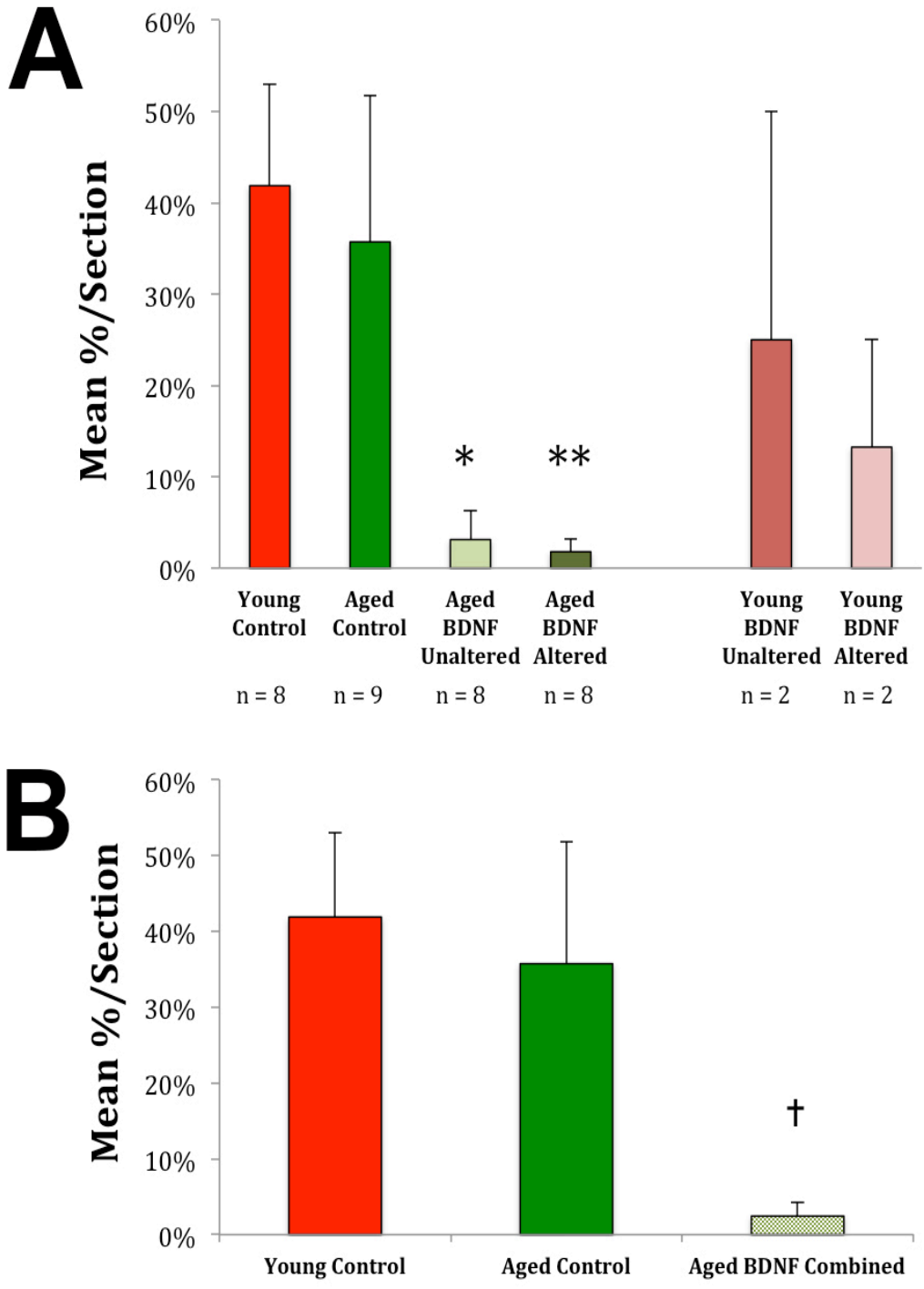

Figure 6: Percentage of BrdU+ cells co-labeled with a neuronal marker.

Figure 6: Percentage of BrdU+ cells co-labeled with a neuronal marker, continued.
(**A**) One-way ANOVA revealed differences among the control groups and aged BDNF treatment groups ($F = 4.45$, $p < 0.01$). Comparing the young and aged controls, there was no observed statistical difference, implying that the proportion of newly born hippocampal cells that adopts a neuronal fate does not change with age, despite the fact that the absolute number of newly born cells is reduced with age (see Fig. 4). Surprisingly, however, BDNF treatment resulted in a reduction in the proportion of cells that express neuronal markers in aged animals compared to aged controls. There was a strong decreasing trend with 92% reduction in subjects with unaltered expression (*$p = 0.08$) and a 95% reduction in subjects with altered expression (**$p = 0.06$) of BDNF. (**B**) One-way ANOVA revealed differences among the three groups ($F = 6.92$, $p < 0.004$) with post-hoc analyses showing that the combined aged BDNF groups is significantly different from the aged control (†$p < 0.01$).

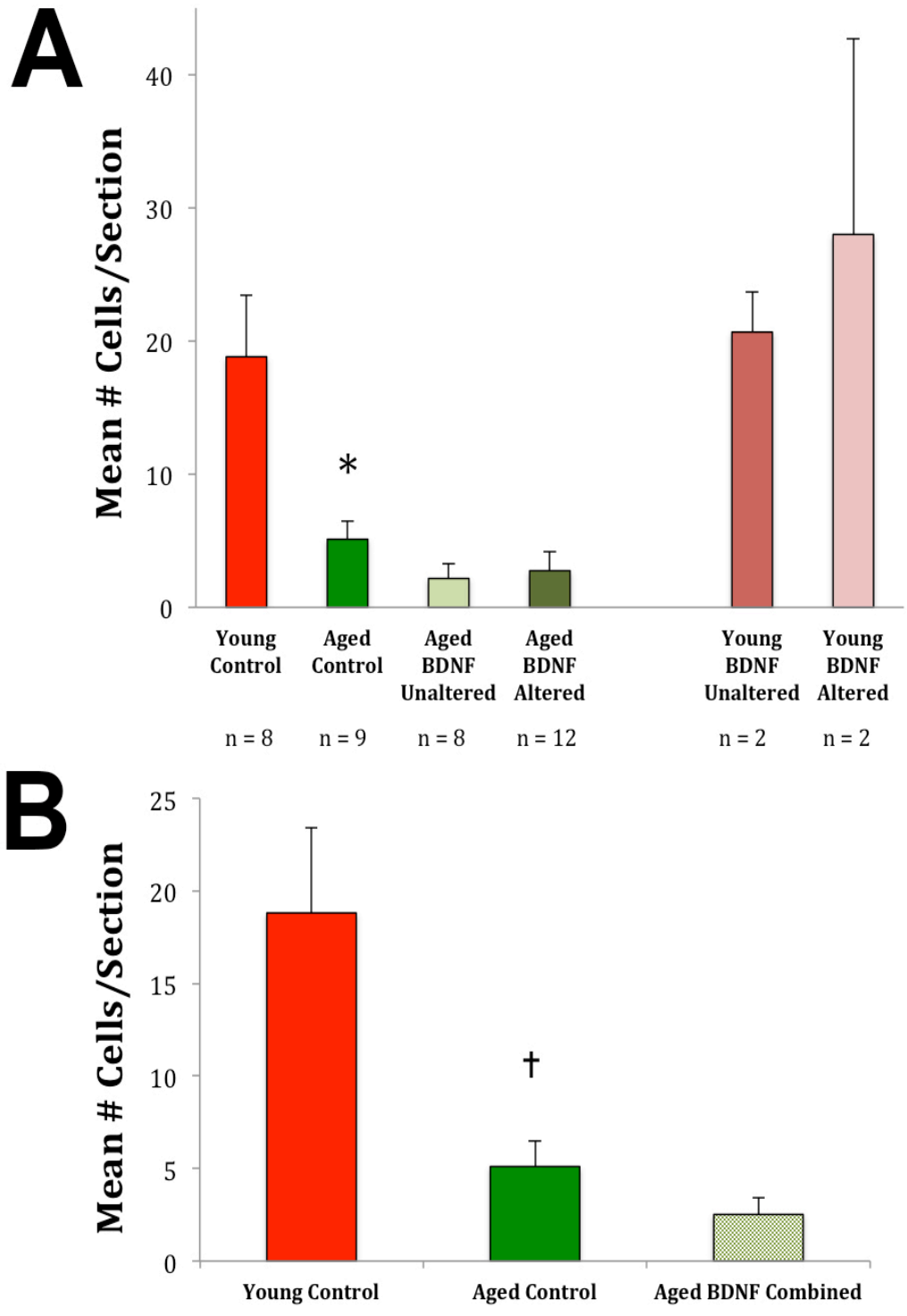

Figure 7: Number of DCX+ cells in the SGZ and GCL.

Figure 7: Number of DCX+ cells in the SGZ and GCL, continued.
(**A**) One-way ANOVA revealed a difference among the two control groups and aged BDNF groups ($F = 10.36$, $p < 0.0001$). A significant reduction in the number of DCX+ cells was observed in the aged controls compared to the young controls (*$p < 0.009$). There was no statistical significance between aged controls and the aged unaltered group ($p = 0.11$) or aged altered group ($p = 0.25$). (**B**) One-way ANOVA revealed a difference among the three groups ($F = 15.98$, $p < 0.0001$). There is a significant reduction in the number of DCX+ cells with aging (†$p < 0.0003$). The combined BDNF aged group showed a 51% decrease in the number of DCX+ cells compared to the aged controls, but this comparison was not significant ($p = 0.13$).

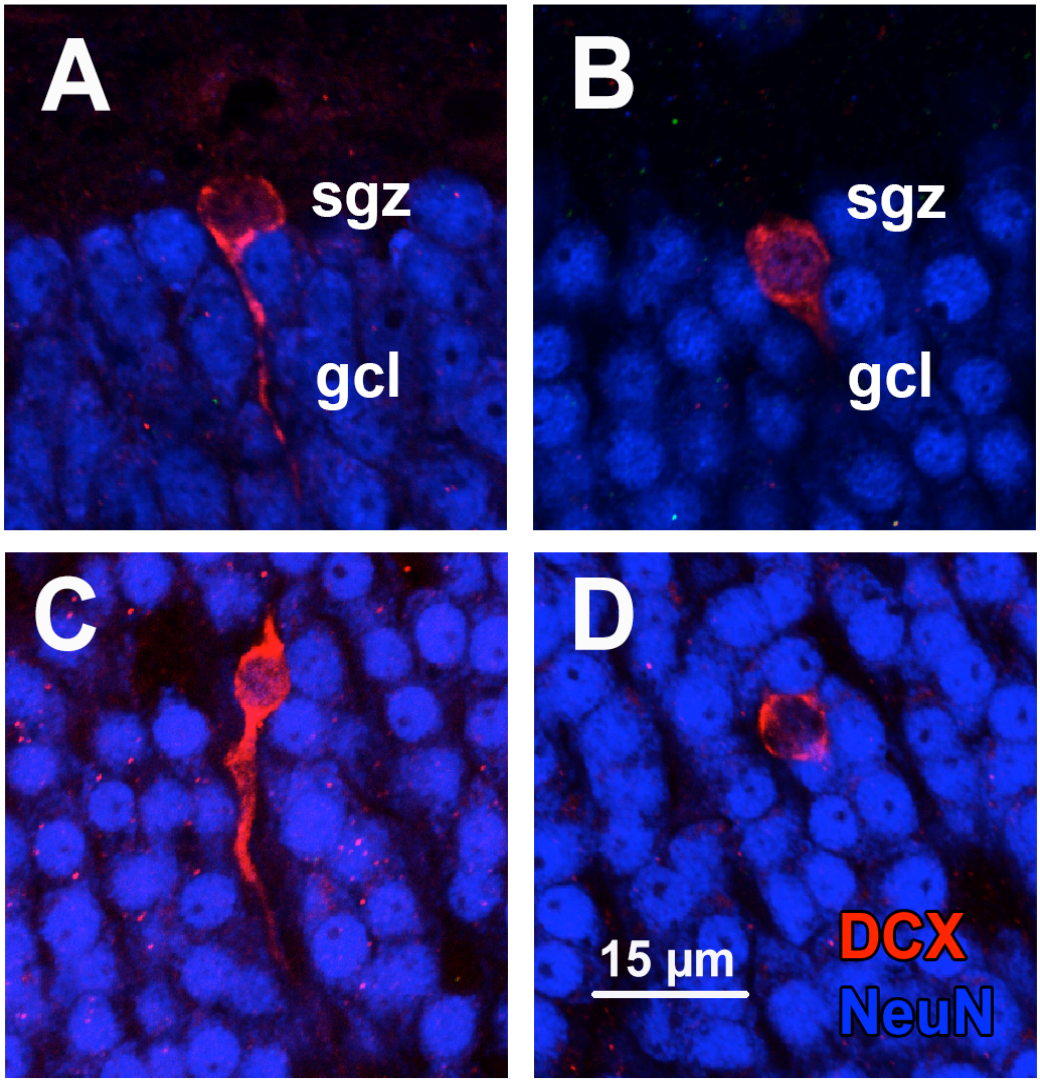

Figure 8: Different qualities of DCX+ cells in the SGZ and GCL. DCX+ cells are categorized by two characteristics, location and branching, as an indication of maturation. Cells categorized as either branching (Fig. 8A, C) or non-branching (Fig. 8B, D). They were also categorized depending on whether they were located in the SGZ (Fig. 8A, B) or GCL (Fig. 8C, D). Mature neurons tend to display processes, and are often localized to the GCL.

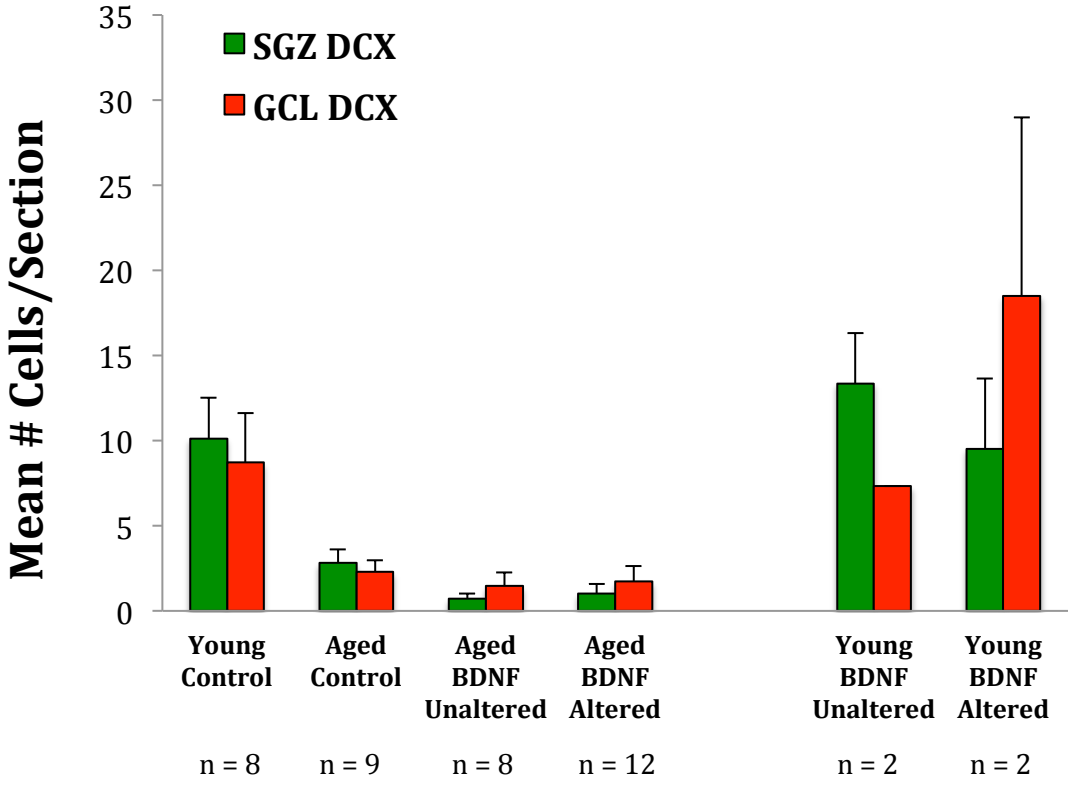

Figure 9: Migration of DCX+ cells in the dentate gyrus. Statistical analysis revealed no interaction effect between the subject group and location of DCX+ cells. There is, however, a decreasing trend in the number of DCX+ cells in the SGZ in the aged treatment groups vs. aged controls with about a 70% reduction in the number of DCX+ cells in the SGZ compared to controls.

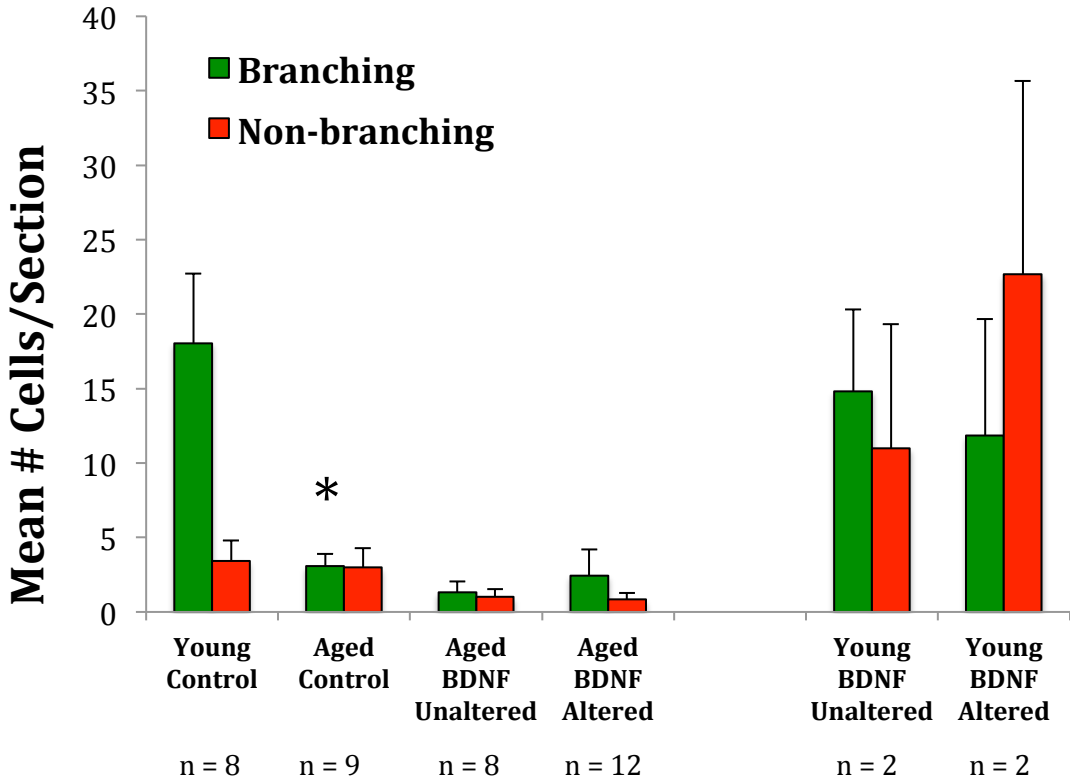

Figure 10: Number of DCX+ cells that show branching. There is an observed decrease in the number of branching DCX+ in aged controls compared to young controls (*$p < 0.0002$). Within the young subjects, there is an observed increased trend of non-branching cells with BDNF treatment compared to controls. This trend showed a 320% increase in young subjects with unaltered expression and 510% increase in young subjects with altered expression.

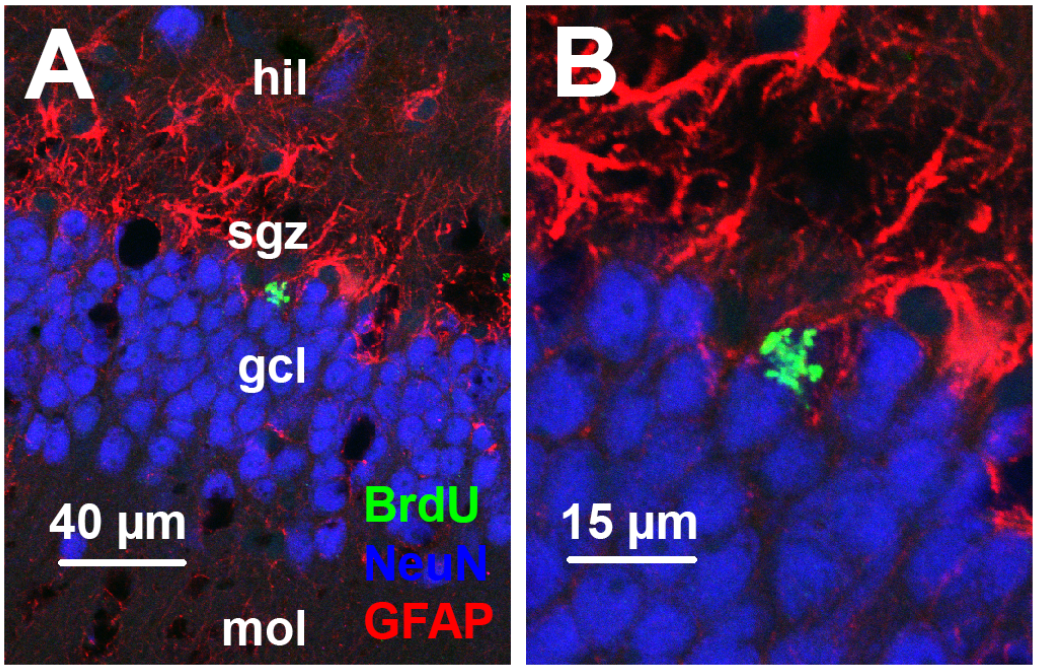

Figure 11: BrdU+ cells do not colocalize with GFAP. Fig. 11A shows the zoomed out image to show the relative position of the cells. BrdU is not colocalizing with GFAP or NeuN in these images. Three sections were taken from each subject with a total of 401 BrdU+ cells located in the SGZ and GCL. GFAP was not found to colocalize with any BrdU+ cell.

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