OVER-EXPRESSION OF BDNF DOES NOT RESCUE SENSORY DEPRIVATION-INDUCED DEATH OF ADULT-BORN OLFACTORY GRANULE CELLS

by

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ABSTRACT

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It is of interest to understand how new neurons incorporate themselves into the existing circuitry of certain neuronal populations. One such population of neurons is that which are born in the subventricular zone (SVZ) and migrate to the olfactory bulb where they differentiate into granule cells. Another area of interest is the role of brain-derived neurotrophic factor (BDNF) on the survival and overall health of these neurons. This study aimed to test whether or not BDNF is a survival factor for adult-born granule cells. Here were utilized a transgenic mouse model over-expressing BDNF under the α-calcium/calmodulin-dependent protein kinase II (CAMKIIα) promoter, and tested its effect on olfactory granule cells under sensory deprived conditions. Results from this experiment indicated that there was no significant difference in cell death or cell survival

when comparing transgenic and wild type animals. We concluded that BDNF is not a survival factor for adult-born granule cells.

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I. INTRODUCTION

Background

Olfaction is arguably one of the most intricate sensory systems. Responses to odorants in the environment can affect mood, emotions, and behavior. To facilitate this, humans are able to discriminate between over 10^{12} distinct odors (Bushdid et al., 2014), and other animals can discriminate even more (Godfrey et al., 2003). For many mammals, olfaction is crucial to survival and plays an important role in finding food, mates, and identifying predators (Young and Trask, 2003). The process of "smell" begins with odorant molecules in the air binding to different odorant receptors that have a unique affinity for individual odorants. These receptors are located on the sensory neurons that line the nasal cavities. Stimulated sensory neurons then relay this information through their axons to the main olfactory bulb, which in turn processes and sends the sensory information to higher olfactory areas, particularly the brain's olfactory cortex (Nagayama et al., 2014).

Anatomy and circuitry of the main olfactory bulb

The olfactory sensory neurons (OSNs) located in the olfactory epithelium are unique in that each one expresses a single type of odorant receptor encoded by a single gene (Figure 1a) (Whitman and Greer, 2009). However, multiple OSNs may respond to different molecular features present in the same chemical odor. When the odorant is

present in the environment, it activates these subsets of neurons that project their axons topographically to target neurons in the olfactory bulb. The OSNs axons make synapses on the dendrites of target cells in sphere-like structures of neuropil known as glomeruli (Whitman and Greer, 2009). The terminals of the OSNs, along with the processes from other cell types that also make synapses within glomeruli, form what is called the glomerular layer (GL) (Figure 1). These neuron types are referred to as juxtaglomerular (JG) cells however; they are actually three morphologically distinct cell types: periglomerular (PG) cells, superficial short axon (sSA) cells, and external tufted (ET) cells (Nagayama et al., 2014).

Periglomerular cells are the most abundant cell type in the GL (Parrish-Aungst et al., 2007). Typically, these cells have dendrites that distribute within only one glomerulus, with their axons terminating in the interglomerular space (Figure 1a). Their activity generates inhibition within the glomerulus via the release of GABA (with a subpopulation of PG cells also being dopaminergic) (Nagayama et al., 2014). Additionally, PG cells can be placed into two different subgroups, Type I and Type II PG cells, depending upon where their dendrites project. Type I PG cells send dendrites to both the olfactory nerve (ON) zone and non-ON zone of the glomeruli and express tyrosine hydroxylase (TH), an enzyme necessary for production of the neurotransmitter dopamine (Baker et al., 1993). These cells are also directly innervated by OSNs. Type II cells however, only send projections to the non-ON zone (Kosaka et al., 1998). PG cells are also one of the two cell types in the olfactory bulb that are generated in adulthood as well as early development, although this usually occurs in small numbers (Whitman and Greer, 2007). Superficial short-axon (sSA) cells are also inhibitory (GABAergic and

dopaminergic) interneurons located within the GL (Kiyokage et al., 2010). However, less is known about these cells and they make up a very small portion of the JG cell population. External tufted (ET) cells are the last type of cells located in the GL. These cells can also be divided into two subgroups. Some ET cells have basal, or secondary, dendrites while some do not. Much like PG cells, external tufted cells generally send their dendritic processes to a single glomerulus. However in contrast to PG cells, ET cells are responsible for excitation within the glomerulus. The majority of ET cells are glutamatergic in nature, though recently a subtype has been found to be both glutamatergic and GABAergic (Tatti et al., 2014).

The next two layers of the olfactory bulb are known as the external plexiform layer (EPL) and the mitral cell layer (MCL) (Figure 1). The EPL contains the cells bodies of tufted cells while the MCL contains cell bodies of mitral cells. These projection neurons extend an apical dendrite into a single glomerulus where it branches to form a tuft that is innervated by OSN axons, therefore odor information going to these output neurons originates directly from sensory neurons in the nasal cavity. They also have axons which exit the bulb and terminate in the olfactory cortex (Nagayama et al, 2010; Igarashi et al., 2010). Both mitral and tufted cells have secondary dendrites that extend throughout the EPL and release glutamate onto granule cells. In addition to the secondary processes of the mitral and tufted cells, the EPL also contains apical dendrites of granule cells (Nagayama et al., 2014). Just deep to the MCL is the internal plexiform layer (IPL) which contains axons of both mitral cells and external tufted cells (Figure 1).

The innermost layer of the olfactory bulb is the granule cell layer (GCL) (Figure 1), which contains the largest population of bulb neurons. Granule cells (GCs) are small,

axon-less interneurons that are predominantly located in the GCL with a smaller number located in the IPL and MCL. These cells have apical dendrites that extend outward into the EPL, and smaller basal dendrites close to the cell body. The apical dendrite begins to branch once it reaches the EPL and forms spines that are the location of reciprocal dendrodendritic synapses with the secondary dendrites of glutamatergic mitral and tufted cells (Nagayama et al., 2014). The dendrite of the mitral cell releases glutamate onto the dendrite of the GC, and the GC releases GABA back onto the mitral cell dendrite at the same synapse. Granule cell bodies and basal dendrites receive centrifugal inputs from various higher brain regions, including olfactory cortex, as well as from mitral cell axon collaterals (Whitman and Greer, 2009). There are six different sub-types of GCs that are categorized based on soma location and dendrite extension. However, all GCs are inhibitory interneurons that release GABA as a neurotransmitter, and it is unknown whether these subgroups perform different specific functions within the olfactory bulb network (Nagayama et al., 2014). Granule cells are unique in that they are the only neuronal CNS population that turns over, and established GCs, dying by apoptosis, are continuously replaced by newborn GCs that can integrate into circuitry throughout adulthood. There are two other populations of adult-born neurons, the PG cells, which also integrate into bulb circuitry, and the granule cells of the hippocampus, but these do not replace lost cells, they are added to the population (Ming and Song, 2005; Whitman and Greer, 2007).

In addition to receiving sensory input, the olfactory bulb also receives various inputs from projection fibers originating from higher brain regions. Although the role of this top-down processing is not fully understood, it appears that olfaction is a result of

intricate interactions between both sensory (bottom-up) and top-down processing (Matsutani and Yamamato, 2008). The projections involved in this top down processing (also referred to as centrifugal afferents) can be categorized into two different groups: neuromodulatory fibers from neurons that do not receive inputs from the olfactory bulb, and fibers from neurons of the olfactory cortex and anterior olfactory nucleus, that do receive inputs from the olfactory bulb.

There are three distinct types of neuromodulatory fibers that send inputs to the olfactory bulb. These fibers originate from specific brain nuclei. The first of these neuromodulators are norepinephrine fibers originating in the locus coeruleus (LC). Although the LC sends axons to numerous locations throughout the brain, about 40% of these fibers terminate in the olfactory bulb in rats (Shipley et al., 1985). Once these fibers reach the bulb, their projections are most densely distributed in the IPL and GCL, but they are also found in the MCL, EPL, and GL. Because of this wide distribution, it is likely that multiple types of neurons located in the olfactory bulb are directly affected by the LC. It appears that the main role of the norepinephrine is to inhibit GCs, which thereby disinhibits mitral and tufted cells (Jahr and Nicoll, 1982). These fibers also directly excite mitral cells (Hayar et al., 2001). It has been speculated that these activities increase mitral cell sensitivity and in turn, increase odor sensitivity and facilitate odor memory. It has also been reported that activation of the α_2-adrenergic receptor may promote survival of new neurons in the olfactory bulb (Veyrac et al., 2005).

The second type of neuromodulatory fibers that innervate the olfactory bulb are serotonergic fibers (5-HT fibers). The bulb receives numerous serotonergic axons from both the dorsal and medial raphe nuclei (McLean and Shipley, 1987a).These fibers are

found in all layers of the olfactory bulb, but they predominantly reside in the GL. Serotonergic input has been observed to play a role in olfactory learning and memory. Specifically, a depletion of these fibers leads to impairment of an odor discrimination tasked learned before the depletion (Moriizumi et al., 1994). Loss of serotonergic afferents also results in atrophy of the olfactory nerve layer and GL, which further signifies the importance of serotonergic inputs to the bulb.

The final neuromodulatory fibers that are present in the olfactory bulb are cholinergic afferents. These fibers mainly originate from the nucleus of the horizontal limb of the diagonal band in the basal forebrain. This area of the brain also contains GABAergic neurons that project axons that terminate in the olfactory bulb. Acetylcholine fibers are found in every layer of the bulb, but are most prominent in the GL. The main targets for these axons are PG cells, mitral cells, and GCs (Castillo et al., 1999). It has been suggested that these fibers play a role in the plasticity of olfactory memory and in the regulation of GC survival (Mandairon et al., 2006; Mechawar et al., 2004).

The olfactory bulb also receives input from areas of the olfactory cortices. These areas include the anterior olfactory nucleus, the tenia tecta, the piriform cortex, the cortical amygdaloid nucleus, and the nucleus of the lateral olfactory tract (Matsutani and Yamamoto, 2008). These inputs terminate mostly in the GCL where they form glutamatergic synapses on granule cells (Balu et al., 2007). These centrifugal projection fibers and the projections originating in the bulb are reciprocal in nature. Areas that receive input from bulb mitral/tufted cell axons typically send centrifugal projections back down to the bulb, and the interplay between these connections plays a role in sensory processing and olfactory learning (Matsutani and Yamamoto, 2008).

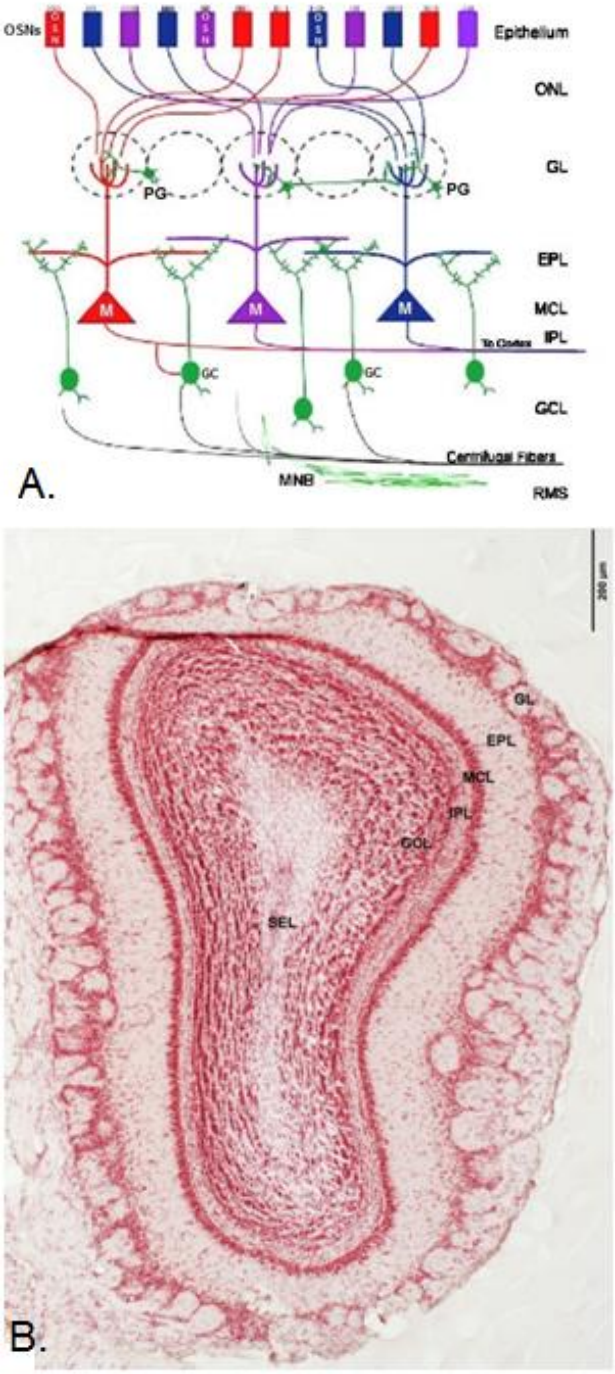

Figure 1: Anatomy and circuitry of the mouse olfactory bulb
A. Circuitry of the olfactory bulb. Modified from Whitman MC and Greer CA (2009) Prog Neurobiol 89:162-195. B. Nissl stain of a coronal olfactory bulb section. The anatomical layers of the bulb are indicated. **OSN**: Olfactory Sensory Neuron, **GL**: Glomerular layer, **PG**: Periglomerular Cell, **EPL**: External plexiform layer, **MCL**: Mitral cell layer **M**: Mirtal cell, **IPL**: Internal plexiform layer, **GCL**: Granule cell layer, **GC**: Granule Cell, **SEL**: Subependymal layer, **MNB**: Migrating neuroblast, **RMS**: Rostral migratory stream.

Adult neurogenesis in the olfactory system

Neurogenesis is the process in which functional neurons are generated from precursor cells (Ming and Song, 2011). Although this predominantly applies to embryonic and perinatal development, it is now known that adult neurogenesis occurs in two distinct areas of the mammalian brain: the subgranular zone (SGZ) of the dentate gyrus of the hippocampus and the subventricular zone (SVZ) lining the anterior lateral ventricles of the forebrain (Figure 2). Adult-born neurons originate from neural stem cells (NSC) in these areas, which by definition, are capable of self-renewal via cell division and are also capable of producing specialized cells through differentiation (Gage, 2000). Adult neural stem cells in the SGZ become neuroblasts that differentiate within the granule cell layer in the dentate gyrus where they mature into hippocampal granule cells (Ming and Song, 2005). New neurons born in the SVZ migrate over a long distance as a stream of neuroblasts, called the rostral migratory stream (RMS) and eventually reach the olfactory bulb were they differentiate into PG cells in the GL or olfactory granule cells in the GCL (Figure 2) (Whitman and Greer, 2009).

Once a SVZ NSC divides asymmetrically to form a neuroblast, it begins to migrate towards the bulb. These immature, migrating neuroblasts are typically bipolar, and during migration, differentiation begins to occur. Once migration has ceased and the cell is in its final location, it fully differentiates into a GC. The differentiated cell will then undergo maturation, which consists of dendritic growth, along with synapse formation and neurotransmitter synthesis (Figure 2).

The SVZ is comprised of four different cell types: Type A, Type B, Type C, and Type E (Figure 3). Type E cells are epithelial cells known as ependymal cells which line the wall of the lateral ventricle and separate it from the SVZ. The cell types of the stem cell lineage include the original neural stem cells (Type B), which are astrocyte-like in nature and are positive for glial fibrillary acidic protein (GFAP) (Doetsch et al., 1999). Dividing B cells give rise to C cells, which are transit-amplifying progenitor cells. These cells continue to divide to generate more type C cells. Type A cells are the neuroblasts derived from dividing type C cells (Lois and Alvarez Buylla, 1994). These cells are doublecortin (DCX) positive, migratory, and are the only cell type to leave the SVZ. However, even as migrating neuroblasts, type A cells remain capable of division (Gritti et al., 2002). They are not all post-mitotic.

Neuroblasts migrate in the RMS (Figure 2), which is formed by the migrating neurons and glial cells (astrocytes) that arrange themselves into channels, or tube-like structures. The tubes serve as migratory pathways that guide the neuroblasts as they travel anteriorly from the SVZ to the olfactory bulb. Interestingly, neuroblasts in the RMS do not use typical migration mechanisms, such as the tangential and radial migration along glial processes that occurs during forebrain development. Instead, they move through the RMS using a process known as chain migration (Doetsch and Alvarez-Buylla, 1996; Lois et al., 1996) (Figure 2). During chain migration, one cell will use the cell ahead of it as a guide and "climb" over that cell. That cell will then serve as the guide for the other cell since it is now in front. This pattern of neuroblasts migrating on each other will continue until the cells exit the RMS in the caudal olfactory bulb. From here

they migrate outward into the GCL (new granule cells), or the GL (new PG cells) (Whitman and Greer, 2009).

Once migration has terminated, the neuroblasts continue to mature and differentiate until they become fully mature neurons (Figure 2). About 10,000-30,000 new cells reach the olfactory bulb every day (Whitman and Greer, 2009). However, about half of these cells are never incorporated into circuitry and die by apoptosis over the next 2-8 weeks (Petreanu and Alvarez-Buylla, 2002). Of the cells that do survive, approximately 95% become granule cells (neurons) while the other 5% become astrocytes (Lledo and Saghatelyan, 2005). When mature, the new cells are functionally and morphologically indistinguishable from older cells present in the bulb (Carleton et al., 2003). In addition to the constant influx of new cells, older GCs in the olfactory bulb are continuously dying at low levels via programmed cell death, particularly in the deeper portion of the GCL (Imayoshi et al., 2008). This death of older cells and incorporation of new cells represents neuronal turnover, rather than neuronal addition, which is why the overall size of the olfactory bulb does not change over time. The ongoing replacement of bulb interneurons throughout the adult life of the animal leads to continuous structural and functional reorganization of olfactory bulb circuitry.

One issue that arises when translating research on the SVZ and olfactory bulb from rodents to humans is that the anatomical organization of the olfactory bulb is different between species. Overall, the human olfactory bulb is comparatively smaller than that of rodents. Although there is evidence that NSCs are produced in the human SVZ, whether they functionally incorporate and what their function is remains controversial. Humans do have an RMS that terminates at the olfactory bulb, but the

general direction and shape greatly differs from that of rodents (Curtis et al., 2007). It also appears that the adult NPCs that migrate along the human RMS may not reach the olfactory bulb, however evidence for this is conflicting (Bergmann et al., 2012; Sanai et al., 2011).

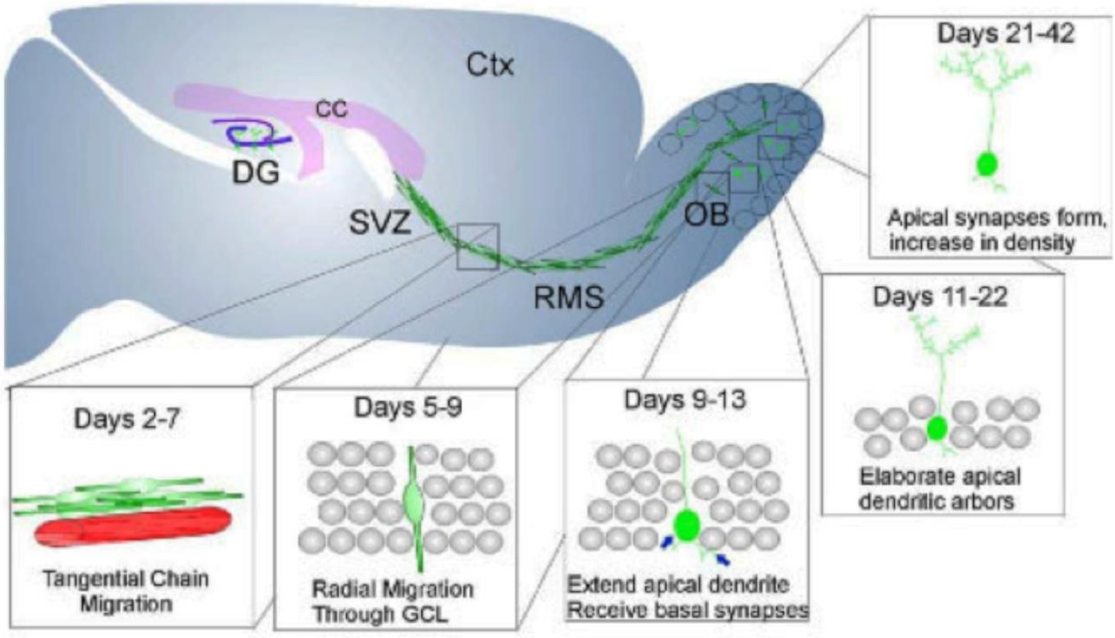

Figure 2: Adult neurogenesis in the rodent brain.
Neuroblasts are born in the SGZ of the dentate gyrus and migrate into the granular layer where they mature into granule cells. Neuroblasts born in the SVZ migrate in the RMS and enter in the olfactory bulb where they differentiate into PG or granule cells. Granule cells begin to extend an apical dendrite into the EPL and their basal dendrites undergo synaptogenesis 9-13 days after birth of the cell. Apical dendrites then arborize and form spines and synapses (from Whitman and Greer, 2009).

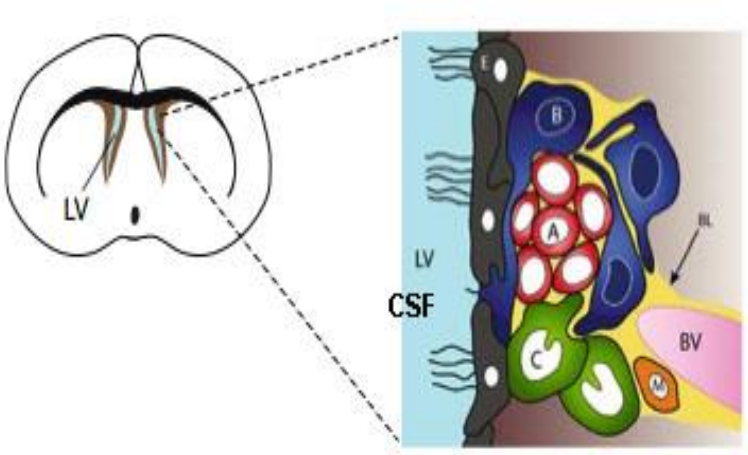

Figure 3: Stem cell lineage in the SVZ.
Type E ependymal cells line the wall of the lateral ventricle. Type B cells are astrocyte-like adult neural stem cells. Dividing B cells generate transit-amplifying Type C cells. C cells give rise to more C cells and Type A cells (migrating neuroblasts). Type A cells leave the SVZ and travel in the RMS into the olfactory bulb. LV: lateral ventricle; CSF: cerebral spinal fluid; BV: blood vessel. From Ihrie and Alvarez-Buylla (2008) Cell Tissue Res. 331:179-191

Maturation and integration of adult-born granule cells

Understanding how adult-born neurons integrate into existing neural circuitry in the olfactory bulb is of great interest in order to understand both the mechanisms and functional significance of adult neurogenesis in this system, as well as a basic understanding of how new neurons can incorporate into adult brain circuitry. The differentiation and maturation of new-born GCs in the olfactory bulb can be outlined in five stages (Petreanu and Alvarez-Buylla, 2002) (Figure 2). Stages 1 and 2 occur 2-7 days after "birth" of the neuron, when they appear as strictly migratory cells, usually bipolar with undifferentiated processes. Stage 3 occurs 9-13 days after birth. At this point the new neuron reaches the GCL and begins to extend a simple, unbranched apical dendrite towards the EPL. Stage 4 occurs 11-22 days after birth and consists of further dendritic development that includes branching. Finally, stage 5 occurs 15-30 days after cell birth

and is characterized by mature features that include dendritic spine formation. Studies have shown that mature-looking granule cells can first be seen at about 14 days after birth of cell age (Whitman and Greer, 2007). Some cells at this point have a multi-branched apical dendrite with immature spine-like protrusions and basal dendrites that are fairly spiny. Spines on the basal dendrites tend to form and mature first while spines on the apical dendrite remain relatively immature until approximately 21 days after birth. It is during this time (2-3 weeks after cell birth) that these adult generated GCs begin establishing synapses with mitral and tufted cell secondary dendrites. At this point, spine numbers peak. By 8 weeks, spine number and density decreases, and the morphology of some spines returns to looking more filopodial rather than mature (Whitman and Greer, 2007). The pruning of spines is thought to indicate activity-dependent stabilization of those spines that are functionally mature, and elimination of those that are not. During the phase of dendritic maturation and synapse formation, between 2 and 8 weeks, about 50% of newly generated granule cells die by apoptosis, with those that functionally incorporate surviving long term, beyond this period (Petreanu and Alvarez-Buylla, 2002; Whitman and Greer, 2007). Because GCs lack axons, their dendritic development is crucial for their successful functional integration.

Brain-derived neurotrophic factor

There are multiple factors that can affect the proliferation, migration, differentiation, and integration of adult born neurons in the olfactory bulb. Such factors include growth factors, hormones, and neuronal activity. One growth factor that has been suggested to affect adult neurogenesis is brain-derived neurotrophic factor (BDNF). BDNF is one of the four classic neurotrophins and has been shown to play a role in

neurogenesis, and neuron survival and differentiation, particularly in some peripheral neuron populations (Barde, 1994; Conner and Dragunow, 1998). In CNS neurons, BDNF gene expression and peptide secretion is activity-dependent, and calcium-regulated (Zheng et al., 2012). There are 9 promoters that control BDNF gene transcription. The BDNF gene itself consists of 8 non-coding exons and one coding exon (denoted exon IX) that are spliced to form one of 22 different mRNA transcripts (Zheng et al., 2012). At least one of these transcripts is preferentially transported into neuronal dendrites for local BDNF synthesis near spines/synapses in adult forebrain neurons in vivo (An et al., 2008). The precursor for mature BDNF peptide is a larger protein known as proBDNF, which binds to the low-affinity neurotrphin receptor $p75^{NTR}$ which promotes cell death and cell cycle suppression (Lee et al., 2001; Teng et al., 2005). When proBDNF is cleaved by enzymes in neurons (furin), the smaller, mature BDNF peptide is generated. When secreted, this binds to the tropomysin-related kinase receptor type B, or TrkB, on cell surfaces (Chao, 2003). The activated form of this receptor tyrosine kinase is auto-phosphorylated. Once BDNF binds and activates TrkB, one of three second messenger pathways will either alter protein function or gene expression in the cell (Purves et al., 2012). The phosphoinositide 3-kinase (PI3)/ protein kinase B (AKT) pathway promotes cell survival and synaptic plasticity, the mitogen-activated protein (MAP) kinase pathway promotes neurite growth and differentiation, and the CaMKII and protein kinase C pathways promote neuronal plasticity (Figure 4) (Purves et al., 2012; Reichardt et al., 2006). BDNF expression is widespread throughout the forebrain. In the adult bulb, levels are very low compared to other forebrain areas like hippocampus, and BDNF mRNA

expression can be detected in scattered juxtaglomerular neurons, and at very low levels in granule cells (Guthrie and Gall, 1991; Conner et al., 1997; Clevenger et al., 2008).

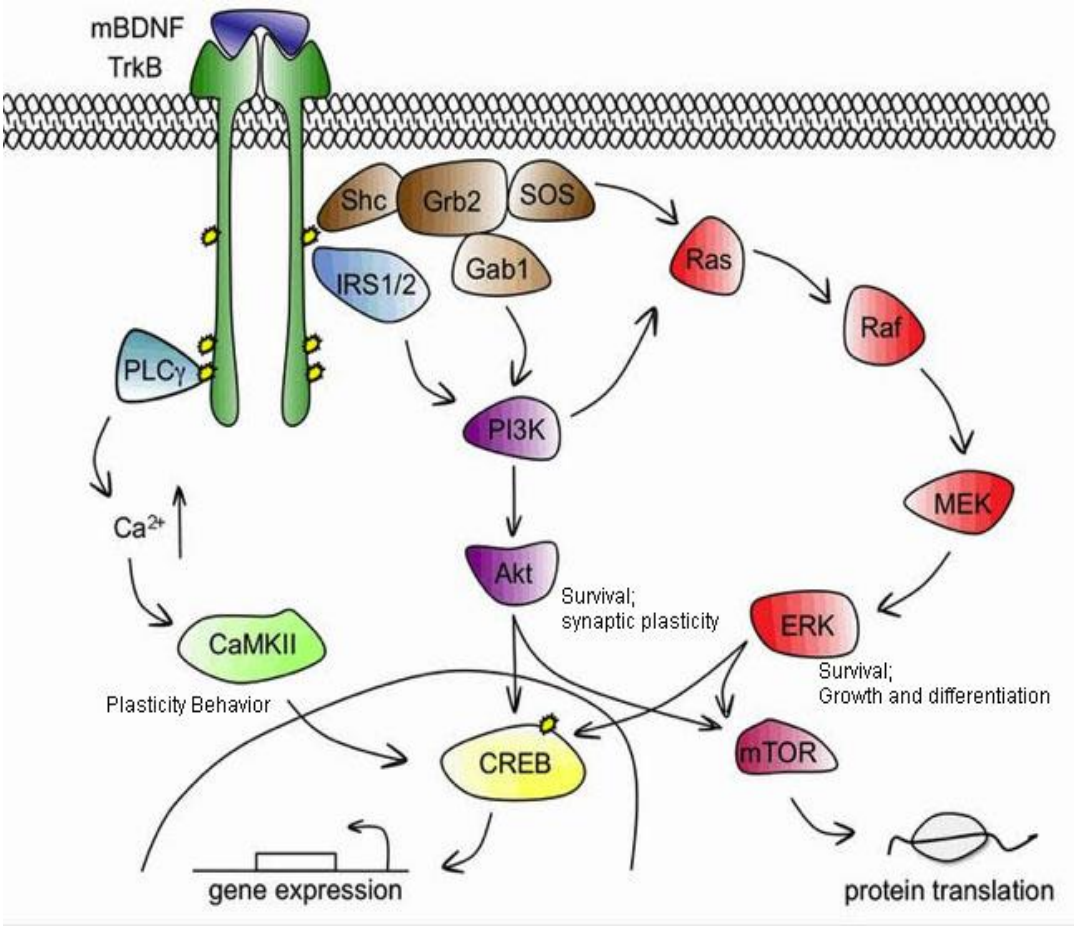

Figure 4:TrkB signaling pathways activated by BDNF.
PLCγ/CaMKII activation of cAMP responsive element binding protein (CREB) promotes plasticity and regulates gene expression. The Map kinase cascade promotes cell growth and differentiation through ERK1/2 signaling. The PI3-Kinase-AkT pathway promotes cell survival and synaptic plasticity (Reichardt, 2006; Cunha at al., 2010). Modified from: Cunha C, Brambilla R, Thomas KL. 2010. Front Molec Neurosci. 3:1-14.

The role of neural activity in olfactory neurogenesis

One factor that has been shown to affect the survival and integration of adult born GCs in the olfactory bulb is sensory activity. Multiple studies have shown that sensory

input via odor stimulation plays a key role in the survival, differentiation, and integration of new olfactory neurons. For example, placing mice in an enriched odor environment (long-term exposure to different odors) has been shown to increase the number of surviving adult-born GCs at 3 weeks after cell birth; measured with bromodeoxyuridine (BrdU) cell labeling (Rochefort et al., 2002). Along with an increase in numbers of cells, there was also an improvement in performance of an olfactory memory task in groups of mice that were subjected to the enriched environment (Rochefort et al., 2002). However, the effects of odor-enrichment are short-term, and the numbers of adult-born neurons in the bulb return to control levels about one month after mice are removed from the enriched environment (Rochefort et al., 2005). Similarly, the increased odor memory seen in the animals under enriched conditions diminishes approximately one month after the enrichment ceases. Adult born PG cells are also affected by odor-enriched environments. Specifically, odor enrichment upregulates synaptogenesis (formation of synapses) in adult born PG cells during the early phases of their morphological development (Livneh et al., 2009).

In contrast to the effects of odor enrichment on the integration of adult born neurons in the olfactory bulb, sensory deprivation inhibits the survival and integration of these cells. Deprivation is typically achieved by a unilateral naris occlusion via cauterization or by inserting a plug into one nasal passage. Although the main focus of sensory deprivation studies is usually the adult-born granule cells, it is important to note that deprivation also inhibits the generation of adult-born OSNs in the olfactory epithelium where cells are continuously generated due to constant environmental stress caused by chemicals, viruses etc that enter the nasal cavity (Kikuta et al., 2015). The

reduction in sensory neuron neurogenesis is due to an increase in the lifespan of the mature sensory neurons, presumably because they are more protected from environmental insults when the nasal passage is blocked. This means that once deprivation occurs, the system is disrupted on multiple levels.

Under deprived conditions, adult born granule cells have a significantly reduced survival rate. This effect is seen when the new cells are 14 to 28 days old (Yamaguchi and Mori, 2005). This is known as the critical period for sensory dependent survival of these adult born cells. Sensory deprivation does not significantly affect stem cell proliferation or migration of adult born neurons, nor does it have a significant effect on long term survival after the cells have passed 1 month of age. This effect (the decreased survival rate) occurs even in cells that have reached 21 days of age before the naris occlusion takes place (Bastien-Dionne et al., 2009). This indicates that the 2-4 week age window is a critical time for the proper, activity-dependent integration of newborn neurons into the existing neural circuitry. In addition to survival effects, sensory deprivation also affects the morphology of adult born granule cells. Naris occlusion has been shown to decrease spine number and spine density along the apical dendrite of adult born GCs (Dahlen et al., 2011). Together this suggests that sensory input and the neural activity it triggers are integral parts of the successful synaptic integration and maintenance of adult born neurons in the olfactory bulb.

Current study

It is of interest to understand how new neurons can incorporate themselves into existing neural circuitry. The mechanisms underlying adult neurogenesis and cell

integration in vivo are of great interest when formulating potential cell therapies aimed at recovery of circuit function following a brain injury or neurodegeneration (Benraiss and Goldman, 2011). If we can understand how new neurons can replace lost neurons and successfully incorporate them into existing circuitry under normal conditions in the olfactory system, this knowledge might be translated into strategies that can be used to facilitate replacement of neurons and remodeling of damaged circuits in the injured CNS. Successfully utilizing this knowledge could lead to developments in treatments for neurological diseases and injuries. As BDNF expression and secretion is known to be activity-dependent, it provides an attractive candidate factor that may mediate some of the effects of neural activity on neuron survival, morphological development and integration (Lessmann and Brigadski, 2009). The current study aims to elucidate whether BDNF assists in promoting the survival of adult born granule cells under normal and sensory deprived conditions.

Previous studies have examined the role that BDNF may play in olfactory neurogenesis in terms of acting on SVZ stem cells; however this is still a controversial area of research because work from different laboratories have produced conflicting results (Zigova et al., 1998; Benraiss et al., 2001; Bath et al., 2008; Galvao et al., 2008, Bergami et al., 2013). Some studies have shown that introduction of BDNF in to the SVZ promotes proliferation, migration, and integration of adult born GCs into the olfactory bulb (Reviewed by Bath at al., 2012). Other studies using similar techniques found that introduction of BDNF into the SVZ did not increase cell proliferation and actually decreased survival of adult born GCs (Galvao et al., 2008). Furthermore, other studies have shown that the conditional knockout of the full length TrkB receptor (TrkB-FL) had

effects on GC morphology but did not affect the survival of these cells (Bergami et al., 2013; Galvao et al., 2008). The survival of neurons without full length TrkB (TrkB-FL) suggests that BDNF is not a survival factor because, in this case, the BDNF does not have a receptor to bind to. Although its role in adult neurogenesis and survival of new neurons in the CNS is not clear, BDNF is well known to promote dendritic spine maturation, plasticity and maintenance in adult CNS neurons (Rauskolb et al., 2010; Kaneko et al., 2012; Vigers et al., 2012). It is possible that an increase in spine maturation could lead to increased survival by promoting structural integration and therefore, that BDNF could be a survival factor through this action.

As stated previously, the current study aims to determine if BDNF could assist in the survival of adult born neurons in the olfactory bulb. To test this, a transgenic mouse model that over-expresses endogenous BDNF under the CAMKIIα promoter was used. These animals express elevated BDNF in the olfactory bulb, an area that integrates adult born neurons and expresses low levels on BDNF under normal conditions. This model is different from the models used in previous studies because the over-expression of BDNF occurs in the bulb rather than in the SVZ, and does not utilize BDNF knockout mice which have been known to display developmental deficits (Ernfors et al., 1994). To test the effects of BDNF on this system we approached the current study in two ways.

First, we observed the effects of elevated BDNF protein under normal conditions. This would test if the over-expression of BDNF decreases the normal turn-over rate of adult born GCs and helps them to survive instead. We carried out the study by utilizing multiple immunohistochemical procedures at different time points to measure levels of

survival and programmed cell death of adult born GCs in both wild type (WT) and transgenic (Tg) mice.

For the second half of the study, mice underwent unilateral naris occlusion in order to induce olfactory sensory deprivation. Olfactory deprivation is a model that is used frequently as it is fairly easy to achieve, is reversible, and one bulb can serve as a control to the other. The same time points and immunohistochemical procedures were carried out in order to determine if the over-expression of BDNF in the olfactory bulb rescued the GC death due to reduced sensory input. BDNF working as a survival factor in a system where cells are normally dying could provide insight into how some new neurons successfully incorporate, possibly how to increase numbers of neurons that survive under unfavorable conditions.

For both of the aforementioned studies, our data suggests that the over-expression of BDNF in the olfactory bulb does not rescue death of adult born neurons, either under normal circumstances or under sensory deprived conditions. We have therefore, concluded that BDNF is most likely not a required survival factor in the adult, rodent olfactory bulb. This is consistent with findings of several other groups, and does not support findings based on studies that administered BDNF to progenitors in the SVZ (Galvao et al., 2008; Bergami et al., 2013; Zigova et al., 1998; Benraiss et al., 2001; Bath et al., 2008).

II. MATERIALS AND METHODS

Animals

All animal procedures were approved by the Florida Atlantic University Institutional Animal Care and Use Committee, and comply with National Institutes of Health guidelines. Transgenic (Tg) mice, maintained on a C57Bl6/J background, were obtained from Jackson Laboratories (strain #006579; Bar Harbor, ME). These mice over-express BDNF in the olfactory bulb (and in other areas in the forebrain) because they express a rat BDNF transgene (that includes the BDNF coding sequence) under the α-calcium/calmodulin-dependent protein kinase II (CAMKIIα) promoter (Huang et al., 1999). The transgene is expressed in addition to the normal BDNF gene, which is under control of the normal gene promoter. Granule cells express CAMKIIα while mitral cells do not. Therefore under this promoter, BDNF expression by olfactory granule cells is highly increased compared to the low expression seen in normal mice (about 5-fold increase in protein; McDole, 2015). The strain was maintained by breeding transgenic males with wild type (WT) females to obtain litters that contained offspring of both genotypes. Genotyping of the offspring was conducted by purifying DNA from tail samples of young mice (18-21 days). The tail samples were placed in individual tubes of proteinase k (20mg/μl) and lysis buffer (1M 0.1M Tris, 5M 0.2M NaCl, 0.5M 5mM EDTA, 20% 0.4% SDS). The samples were vortexed and left overnight at 56˚C. The next day, the samples were purified using an isopropanol precipitation procedure that isolates

the DNA. To do this, samples were spun down at 8,000 rpm for 3 minutes and the supernatant was extracted into a new tube. Next, 500µL of isopropanol were added to the supernatant, mixed, and left to sit for 5 minutes. The precipitate from the mixture was removed and transferred into 1000µL of 70% ethanol. The ethanol and precipitate were spun down at 8000 rpm for 3 minutes, the ethanol was removed, and the precipitated was left to dry. Once the sample was dry, 100 µL of Tris-EDTA (TE; pH=8, pre-warmed to 55˚C) were added and votexed vigorously to obtain a sample of isolated DNA in TE. The concentration (ng/µL) of each sample was measured and recorded using a spectrometer. Based on the concentrations obtained, 100ng of isolated DNA mixed with sterile water (to equal 5µL total) was used to perform polymerase chain reaction (PCR) amplification of the DNA. The DNA was added to 25µL of 2X Taq polymerase mix (dNTPs, buffer, Taq DNA polymerase, $MgCl_2$, New England Biolabs, Inc.), 17µL of sterile water, and 1.5 µL each of two different primers (5′-GTGAAGGAACCTTACTTCTGTGGTG-3′ and 5′-GTCCTTGGGGTCTTCTACCTTTCTC-3′). The primers were specific for the identification of the BDNF transgene. The parameters for the PCR cycling were 94 °C-30 s, 55 °C-30 s, 72 °C-45 s for 30 cycles. The PCR product was mixed with a loading dye (10µL product, 2µL 6X loading dye) and loaded into a 5% agarose gel for electrophoresis with positive and negative controls. Gels were imaged on a Licor Odyssey Gel and Blot Imaging system to identify mice that expressed the BDNF transgene.

Bromodeoxyuridine (BrdU) Injections and Unilateral Naris Occlusion

Mice were injected intraperitoneally (i.p.) with a single 150 mg/kg dose of BrdU at 7.5-8 weeks of age. BrdU is a thymidine analog that incorporates into the DNA of

mitotic cells. To obtain the solution, 20 mg of BrdU was dissolved into 2ml of sterile saline and 14µl sodium hydroxide (NaOH) and allowed to mix in a sonicator bath for 45 minutes at room temperature. Twenty-four hours post injection, some of the mice underwent unilateral naris occlusion. The 24 hours between BrdU injection and naris occlusion ensures that the BrdU has fully incorporated into the stem cells and early cell development is underway before the sensory deprivation. The animals were anesthetized using a ketamine/xylazine cocktail (100 mg/kg ketamine; 10 mg/kg xylazine; i.p.). The right naris was then plugged with a small piece of polyethylene tubing (PE50, approximately 4 mm long) filled with dental paraffin wax and beveled at one end (Cummings et al., 1997). The nasal passage was first moistened with sterile eye lubricant which allowed the plug to be inserted easily using forceps. Control mice were anesthetized using the same ketamine/xylazine cocktail but did not receive the occlusion. Mice were returned to the viviarium after fully awake and mobile, and were checked daily for normal respiration, eating, and overall health.

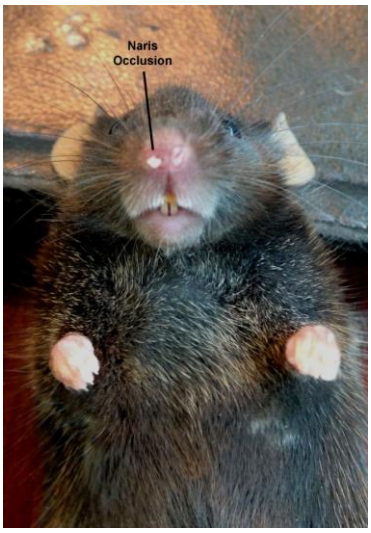

Figure 5: Anesthetized C57Bl6/J mouse after unilateral naris occlusion procedure.

Enzyme-Linked Immunosorbent Assay (ELISA)

Fresh bulb tissue from WT and Tg mice (2 WT control, 4 WT occluded, 2 Tg control, 4 Tg occluded) were collected to carry out BDNF ELISA quantification using Promega's EMax kit (Promega, Madison, WI). The animals were anesthetized and occluded using the procedures described above. Four weeks post-occlusion the mice were euthanized with sodium pentobarbital (150 mg/kg; i.p.), decapitated, and individual olfactory bulbs were dissected and frozen on dry ice. Bulbs contralateral to the occluded naris in deprived mice serve as internal controls for the deprived bulb. The bulbs were stored at -80˚C until needed for the assay. Each individual bulb was homogenized with a cocktail of lysis buffer (#9803, Cell Signaling Technology, Danvers, MA, USA), 1mg/ml complete protease inhibitor, and 1 mg/ml complete phosphatase inhibitor (Roche Life Sciences, Indianapolis, IN, USA). The lysates were then centrifuged for 20 minutes at 4˚C (14,000 rpm). Supernatants were collected and the protein concentration was measured using a Qubit assay (Invitrogen, Carlsbad, CA, USA). Samples were stored overnight at -80˚C. The same day, the ELISA plate was coated with 100 µL per well of Anti-BDNF mAb in carbonate coating buffer (pH=9.7, 0.025M sodium bicarbonate, 0.025 sodium carbonate; dilution 1:1000). The wells were covered with a plate sealer and incubated overnight at 4˚C without shaking. The following day each protein sample was diluted with lysis buffer and 1:5 Dulbecco's phosphate-buffered saline (DPBS, pH=7.35, 3.4 mM potassium chloride, 137mM sodium chloride, 1.5mM potassium phosphate monobasic, 9.8mM sodium phosphate dibasic, 0.9mM calcium chloride dihydrate, 0.5mM magnesium chloride hexahydrate) so that protein concentration was equal to 2µg/µl for each sample. The samples were acidified by adding 5µL of hydrochloric acid

(HCl). Samples were vortexed, spun down, incubated for 15 minutes at room temperature, and checked for a pH of less than 3.0. The process was repeated with 5µL of NaOH and checked for a pH around 7.6. The coated plate was washed once with Tris buffered saline with Tween® 20 (TBST; 20mM Tris-HCl, 150 mM sodium chloride (NaCl), 0.05% tween® 20) and 200 µL of Block & Sample 1X buffer (1:4 in deionized water) were added to each well using an automatic pipettor and incubated for one hour at room temperature with no shaking. The plate was then rinsed with TBST (one time) and 100µL of sample and standards were added to the wells in duplicates. To generate the standards, a dilution of 1:2,000 from the provided BDNF protein standard in Block & Sample 1X Buffer was made. Serial 1:2 dilutions of 100µL per well were then performed down the plate in the columns assigned to the standard curve, creating a concentration range of 0-500 pg/mL. Standards and protein samples were incubated for 2 hours at room temperature with shaking at 400rpm. Following the incubation, the plate was rinsed 5 times with TBST and 100µL of Anti-Human BDNF pAb (1:500) were added to each well to incubate over night at 4˚C with shaking. The next day, the plate was washed 5 times with TBST and 100µL of diluted Anti-IgY horseradish peroxidase (HRP) conjugate (1:200) was added to each well to incubate for one hour at room temperature with shaking (400rpm). Following incubation, the plate was rinsed 5 times with TBST. For color development, 100µL of TMB One Solution were added to each well and incubated at room temperature for 10 minutes with shaking (400rpm). During the incubation a blue color formed. The reaction was stopped by adding 100µL of 1N HCl to each well (the color changes to yellow as a visible sign of the reaction). The samples' absorbance at 450 nm were measured using a SpecraMax M5 plate reader (Molecular Devices, Sunnyvale,

CA, USA) and the BDNF concentration of each sample was calculated using SoftMax Pro software.

Tissue Preparation

At specific time points after BrdU treatment and naris occlusion (12 days, 28 days, and 56 days), mice were euthanized with sodium pentobarbital (150 mg/kg; i.p.), and transcardially perfused with ice cold phosphate-buffered 0.9% saline solution (PBS, pH= 7.4) followed by 4% paraformaldehyde (pH=7.4, ~200 mls). The brains were carefully dissected and post-fixed in the same fixative over night (ON). After postfixing, the brains were placed in 30% sucrose in 0.1 M phosphate buffer (PB, pH=7.3-7.4) for cryoprotection for 3-5 days. The brains were then embedded in 10% gelatin and all deprived bulbs (right) were denoted by a notch made by a razor blade in the gelatin surrounding the deprived bulb. The gelatin with brains then underwent brief fixation and cryoprotection (overnight, and for 2 nights respectively) once more before being frozen by immersion in 2-methylbutane at -45˚C, and stored at -80˚C. The brains were cut into serial coronal sections of 1 in 5, at 30µm thickness using a cryostat set at -20˚C. Sections were collected free-floating into PB (pH= 7.3-7.4) and stored at 4˚C.

As previously mentioned, euthanasia of the animals occurred at three different time points after BrdU injection (Figure 6): 12 days, 28 days, and 56 days. There were four occluded mice and two control (non-occluded) mice per genotype at each time point (12 per time point; 36 total, plus up to ~10 extra in case of incomplete occlusions, poor perfusions etc). Results of cell counts of BrdU+ cells in the GCL (described below)

compared the percentage of the starting population lost over time in the WT and Tg mice, with and without (controls) sensory deprivation.

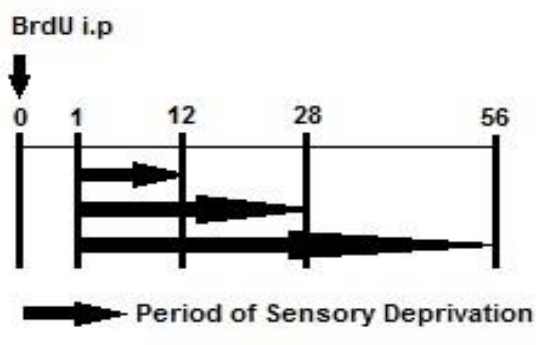

Figure 6: Experimental time line
Tissue collection for counts of new GCs were done at the indicated times after BrdU treatment. Deprivation began one day after BrdU treatment**.**

Immunohistochemistry

Tyrosine Hydroxylase (TH) immunostaining:

Bulb sections (1 in 5) were rinsed with 0.1 M PB (pH = 7.3-7.4) and then blocked with 5% normal goat serum in 0.1M PB for one hour at room temperature. Sections were then transferred to a mixture of a rabbit anti-TH antibody (1:1000; Millipore, cat#AB152 Temecula, CA), 3% normal goat serum, and 0.3% Triton A-100 in 0.1M PB and left to rotate at 4˚C overnight. The following day, sections were rinsed in 0.1M PB and transferred to an AlexaFluor594-conjugated anti-rabbit secondary IgG antibody (1:1000, Life Technologies, Carlsbad, CA) in 0.1M PB and left to incubate for one hour at room temperature. The sections were then rinsed one last time and mounted onto gelatin-coated slides. Sections were imaged at 10X and 20 X magnifications using a Zeiss LSM 710 confocal microscope. Imagining was for the purpose of visualizing a qualitative decrease

in TH immunoreactivity in the glomerular layer in order to confirm deprivation. It is known that the expression of TH mRNA and protein is activity-dependent, and naris occlusion is well-known to decrease this expression in dopaminergic PG cells in the ipsilateral, deprived bulb (Baker et al., 1993).

Bromodeoxyuridine Immunostaining

Two separate immunostaing procedures were performed. The first was an immunoperoxidase staining protocol that labels all BrdU+ cells in tissue sections permanently with a brown reaction product. One in 5 serial sections per animal were collected in order, individually, and every other section was taken in order to create a 1:10 serial set of sections. The sections were rinsed in 0.1M Tris buffered saline (1X TBS, pH= 7.4) and subsequently quenched with 0.6% hydrogen peroxide (H_2O_2) for 8 minutes to quench endogenous peroxidase. The tissue was rinsed once again in 1X TBS. Sections were then placed in a 1:1 mixture of 50% formamide/2X saline sodium citrate (SSC, pH=7.0) for 30 minutes at 65˚C and then rinsed with 2XSSC at room temperature. The DNA was then denatured by incubating the sections 2N HCl for 30 minutes at 37˚C. Immediately following incubation, sections were transferred into 0.1M sodium borate (pH=8.5) to neutralize the acidity from the 2N HCl. Sections were then rinsed in 1X TBS and blocked with 5% normal rabbit serum in 1XTBS for one hour at room temperature. Sections were transferred into a mixture of rat anti-BrdU antibody (1:500; Accurate Chemical & Scientific Corporation, cat#OBT0030G Westbury, NY), 0.3% Triton X-100, and 1X TBS and left to incubate overnight at 4˚C. The following day sections were rinsed with 1X TBS and transferred into a mixture of biotinyated anti-rat secondary IgG (1:200; Vector Laboratories, Burlingame, CA), 3% normal rabbit serum, and 1X TBS for two

hours at room temperature. The tissue was rinsed in 1X TBS and transferred into an avidin-biotin-HRP complex (Vector Elite ABC kit) in 1XTBS for 1.5 hours at room temperature. The reaction product was then developed using Vector Labs Impact DAB kit (containing diaminobenzidine and hydrogen peroxide). This technique permanently labels all BrdU+ cells in a given section. The sections were mounted onto gelatin-coated slides, air dried, dehydrated with ethanol, counterstained with neutral red Nissl stain (to visualize the cell layers), and cover-slipped with Fisher Permount. Five sections from each animal at each time point were counted (starting from the first appearance of the accessory olfactory bulb (AOB) and taking each subsequent, anterior sections) in order to obtain the BrdU+ cell numbers and for each mouse. All BrdU+ cells in the granule cell layer were counted using an Olympus AX70 light microscope at 20X objective magnification. Each section was counted twice and averaged together for a section total. Section counts from each animal (separated right and left for occluded animals) were added together for a final cell count per bulb per animal. Numbers were then adjusted to reflect the total number of *neurons* calculated from the BrdU/NeuN cell counts (detailed below).

The second procedure used was immunofluorescent BrdU/NeuN co-labeling. This immunostaining was carried out using the same protocol as the immunoperoxidase staining with a few exceptions. The sections were blocked in a mixture of 10% normal goat serum, 0.3% Triton X-100 and TBS and left to incubate over night at 4˚C. Primary antibodies included rat anti-BrdU (1:500, as above) and mouse anti-NeuN (1:500; cat #MAB377, Millipore/EMD, Temecula, CA) and were left to incubate for three nights at 4˚C (NeuN is an endogenous marker located in the nuclei of mature neurons).

Fluorescent secondary antibodies were AlexaFluor 488-conjugated anti-rat (1:1000) and AlexaFluor 594-conjugated anti-mouse (1:1000) IgG antibodies. Sections were imaged at 20X objective magnification using a Zeiss LSM 710 confocal microscope and select portions of the GCL of each section (4 images per section; one from dorsal, medial, lateral, and ventral aspects, 150 x 250 microns each) were captured from a minimum of 4 bulb sections, left and right (8 samples, 1 in 5). Per individual bulb section, the total GCL aea sampled was $0.15mm^2$, making the total GCL area sampled per bulb per animal $0.6mm^2$ (4 sections at $0.15mm^2$ each). The sections imaged began at the most caudal section before the appearance of the AOB, followed by the next 3 consecutive sections moving anteriorly. Sections were mounted onto gelatin-coated slides, air-dried, cover-slipped with VectaShield mounting medium (without DAPI), and stored at 4˚C. Co-labeled cells from each section were counted using NIH ImageJ software (with cell counter plug-in) to denote if cells were co-labeled or not (De Vos, 2010; Rasband, 2010). Cells labeled with both NeuN (green) and BrdU (red) were considered neurons while cells labeled with BrdU only were considered glial cells. Cells that were labeled with NeuN only were not counted. A percentage was then calculated based on how many adult born (BrdU+) cells were neuronal (NeuN+) and how many cells were only BrdU+ (glia). This percentage was then applied to the total BrdU+ (peroxidase) counts to determine a final number of adult born granule cells.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)

Levels of apoptotic cell death were assessed with TUNEL, which labels the terminal end of fragmented nuclear DNA. This assay was performed on 9 to 12 sections per animal in the 28 day time point (both left and right bulbs).Sections were initially

rinsed in 0.1M PB (pH7.3-7.4) and treated with 3% hydrogen peroxidase in 1X PBS (pH=7.4). The sections were then rinsed again in 0.1M PB and transferred to 0.05M PB to mount onto Superfrost-plus slides. Slides were dried at room temperature and then placed in an incubator overnight at 37˚C. The following day, the sections were circled with a PAP pen and rehydrated in Copllin jars with fresh ethanol of 100%, 95%, 70%, and 50% concentration respectively for 5 minutes each. The sections were then rinsed with 1X PBS for 15minutes. Following rehydration, the slides were blotted and laid in a plastic container. Proteinase K (50µg/mL) was added to each section (~35-40µL) for 20 minutes at 37˚C for permeabilization and then rinsed in 1X PBS. The slides were then blotted and laid in the container again. A TUNEL reaction mixture [TUNEL stock buffer (pH=7.2, 25mM Tris–HCl, 200mM Na cacodylate, 0.25mg/mL BSA), 1mM cobalt chloride, 200U/mL terminal deoyneucleotidyl transferase (New England Biolabs, #M0315), 13 µM biotin-14-dATP (Life Technologies, #19524-016)] was added to each section (~35-40 µL) and incubated at room temperature for 15 minutes and then at 37˚C for 2.5 hours. After rinsing in 1X PBS the slides were blotted and blocked in 2% bovine serum albumin (BSA, ~40-50 µL per section) for 30 minutes at room temperature. The slides were then rinsed and blotted once more, and ABC from the Vector Elite Kit (1:100) was added to each section (~40-50 µL) and incubated overnight at 4˚C. The next day, the slides were brought to room temperature and developed one at a time using the Vector Impact- DAB kit. Starting from the posterior portion of the bulb (before the start of the AOB) 5 sections from each animal were used to count TUNEL+ nuclei at 20X objective magnification using an Olympus BX41 light microscope. All TUNEL+ cells

within the GCL were counted for each section, and section counts were added to determine a total cell count per bulb per animal.

GCL Volume Estimations

One in 5 serial sections per animal from the 56 day time point were collected in order, individually until right before the appearance of the AOB. These sections were mounted onto superfrost-plus slides, dried at 37˚C, dehydrated using ethanol, and stained with neutral red to define the bulb cell layers. Tissue was examined and calibrated images of sections were collected at 4X objective magnification using an Olympus AX70 light microscope. Following this, the GCL of each section was outlined using NIH ImageJ software using the MCL and IPL as the defining boarder, and the volume of the GCL in the bulb was estimated. This estimate was calculated using a formula that uses known section thickness (30μm), averages the volume of the intervening sections, and then estimates total volume by adding these volumes as described in Cummings et al. (1997) and Mirich et al. (2002).

Statistical Analysis

Data from this experiment are shown as group mean values ± the standard error of the mean (SEM). Analysis was performed using GraphPad InStat software. For comparisons between right and left bulbs of occluded (experimental) animals, paired, two-tailed t-tests were performed. For comparisons between WT and TG controls, or right bulbs of occluded animals and bulbs of control animals, unpaired, two-tailed t-tests were performed. Data were considered statistically significant at a probability of $p<0.05$. All bar graphs of data (in "Results") illustrate the group mean values ± SEM.

III. RESULTS

BDNF protein levels remained high in deprived bulbs of Tg animals

ELISA was used to show that BDNF levels in the Tg mouse bulb do not decrease with sensory deprivation, and remain higher than levels in WT mice (McDole et al., 2015). Although normal BDNF expression is activity dependent under the normal promoter, under the CAMKIIα promoter, BDNF transgene expression is not down-regulated under conditions of low neural activity (Gianfranceschi et al., 2003). Therefore, olfactory deprivation should not reduce overall levels of BDNF in the olfactory bulbs of the occluded transgenic mice, allowing us to test its ability to affect newborn GCs.

The results of this study were similar to those of a previous study in our lab showing that endogenous bulb BDNF levels were significantly increased in Tg animals versus their WT counterparts (McDole et al., 2015). As expected, olfactory deprivation did not have a significant effect on the levels of BDNF when comparing bulbs ipsilateral to the naris occlusion (IBs) and contralateral, non-deprived bulbs (CBs) in TG animals (Figure 7). This indicates that BDNF remained significantly increased due to transgene expression and therefore, allowed us to test if BDNF act as a survival factor for adult born GCs. There was also no significant difference in deprived bulbs of WT animals when compared to their controls as well (Figure 7).

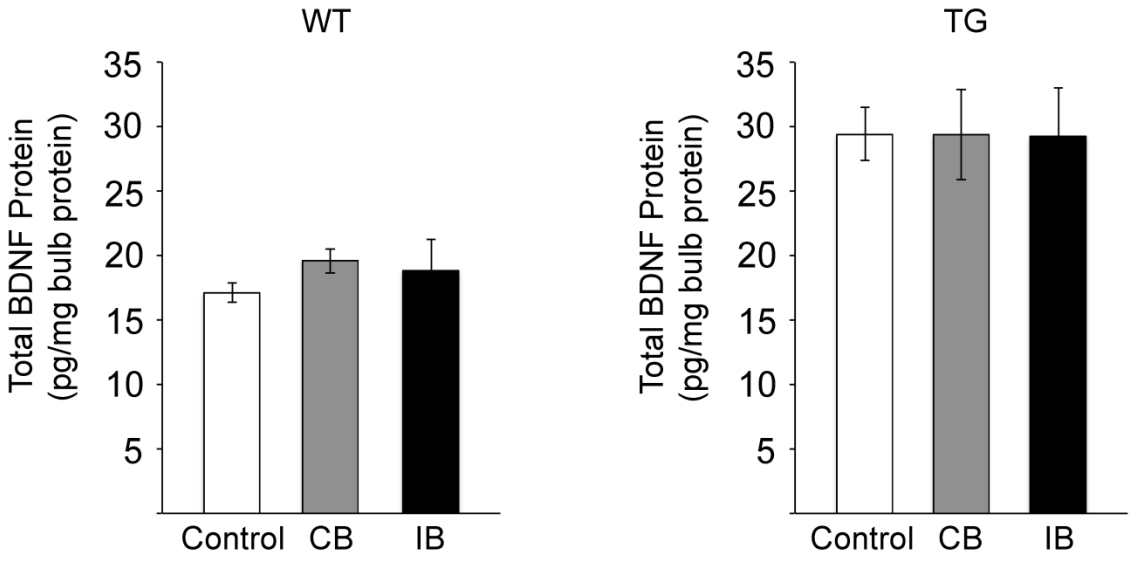

Figure 7: Unilateral naris occlusion does not reduce expression of BDNF in Tg mice
Quantification of total bulb BDNF protein content showed no significant difference between control, contralateral (CB), or ipsilateral (IB) deprived bulbs in both WT and Tg animals.

Sensory deprivation decreased TH expression the dopaminergic neurons

Before other histochemical procedures were performed, a procedure for neuronal TH immunolabeling was carried out. Tyrosine hydroxylase is the rate-limiting enzyme for catecholamine synthesis and it is expressed by dopaminergic PG cells (Daubner et al., 2011; Nagayama et al., 2014). This expression is activity-dependent and naris occlusion is well-known to decrease TH mRNA and protein expression in the ipsilateral, deprived bulb (Baker et al., 1993). Immunostaining showed a dramatic loss of TH immunoreactivity by PG and some tufted cells in the GL following successful sensory deprivation in both WT and TG (Figure 8). This procedure was therefore a qualitative

way to determine if sensory stimulation was effectively eliminated in a particular animal. There was no qualitative difference between TH expression in WT versus TG animals.

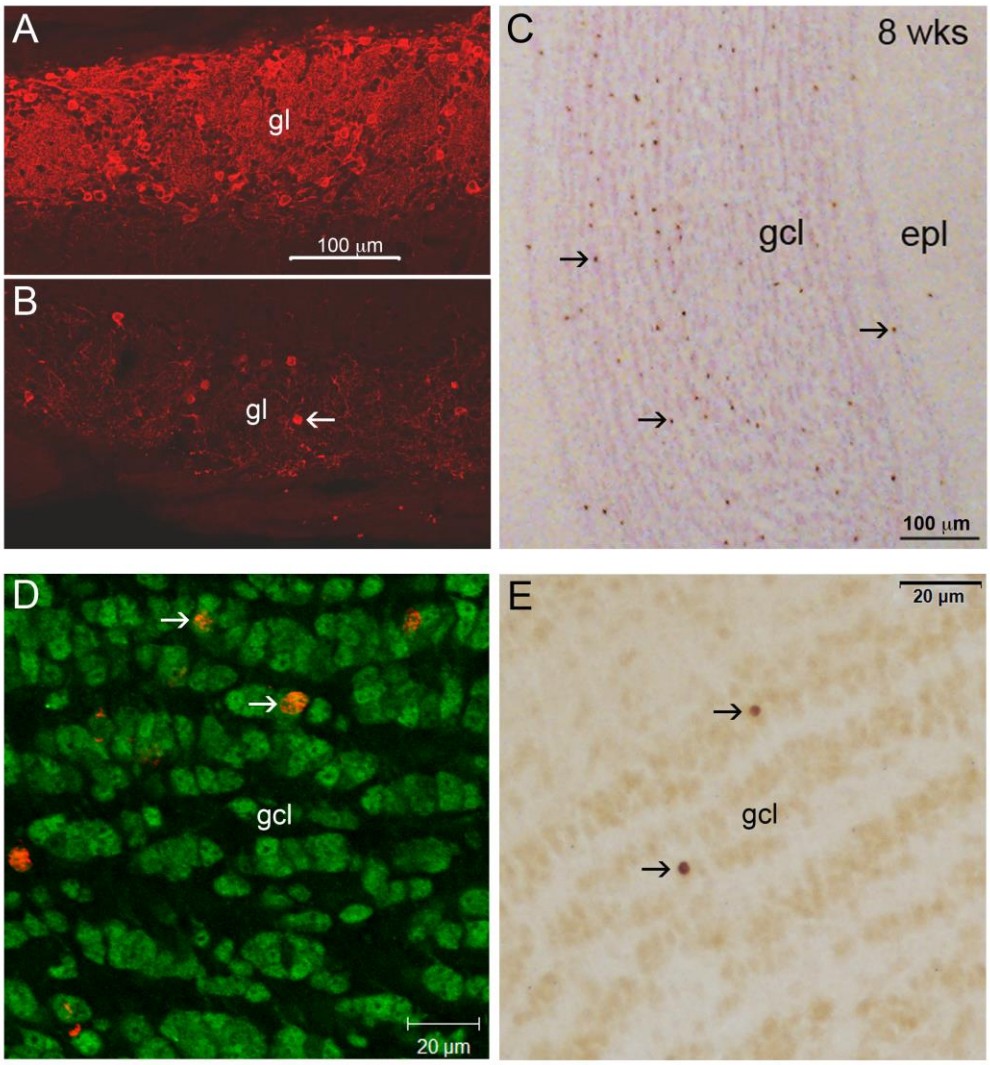

Figure 8: Photomicrographs of immunostaining
(A) Photomicrograph of TH staining in the glomerular layer (gl) of a normal bulb and (B) staining in the gl of a sensory deprived bulb at 56 days post-occlusion. The arrow indicates a remaining TH+ PG cell body. (C) BrdU+ cells (stained using immunoperoxidase procedure) in the granule cell layer (gcl) of a sensory deprived bulb at 56 days post-occlusion. Arrows indicate BrdU+ nuclei. Epl; external plexiform layer. (D)BrdU/NeuN immunofluorescent staining in the gcl of a sensory deprived bulb at 12 days post-occlusion. Arrows indicate nuclei that a co-labeled for BrdU and NeuN. (E) Photo of TUNEL+ cells in the gcl. Arrows indicate nuclei positive for TUNEL (fragmented DNA).

BDNF over-expression did not increase numbers of BrdU+ cells

It is known that some adult-born cells from the SVZ differentiate into glial cells (~5%; Lledo and Saghatelyan, 2005). These cells would incorporate the BrdU upon injection and would be labeled during immunoperoxidase procedures (Figure 8c). Therefore, it is impossible to definitively know whether or not a cell is a neuron or a glial cell when using only BrdU immunoperoxidase staining. NeuN (Neuronal Nuclei protein) is a mature neuronal marker and is only expressed in fully differentiated neurons, including bulb GCs. Therefore, co-labeling of cells indicated that the new cells were GCs and not glia (Figure 8d). The ratio of neurons to glia (labeled only for BrdU) was then used to calculate the total numbers of BrdU+ neurons from the immunoperoxidase stained sections to determine the final numbers (minus glia).

The first time point analyzed was at 12 days post-occlusion. Here, the bulk of the new GCs have arrived in the bulb. Most new granule cells exit the RMS and distribute in the GCL at 10-14 days after birth. This provided a "starting population" of new cells, when their numbers were high and they were not yet sensitive to the effects of deprivation. Immunofluorescent staining indicated that 98% of the labeled population was comprised of neurons at this time point and final numbers were adjusted to reflect this. As expected, paired, two tailed t-tests revealed no significant difference in numbers of counted adult born GCs between IBs and CBs in both WT (IB=3104.0 ± 175.8, CB=3112.9 ± 12.2, $p=0.96$, $t_{(3)} =0.05$) and Tg (IB=2572.9 ± 138.6, CB=2665.2 ± 154.6, $p=0.64$, $t_{(3)} =0.52$) animals or between IBs and controls (WT (Control=2910.3 ± 153.3, $p=0.44$, $t_{(5)} =0.83$), Tg (Contol= 2994.2 ± 101.7, $p=0.06$, $t_{(5)} =2.45$); Figure 9). There was also no significant difference between cell counts in WT versus Tg control animals

($p=0.67$, $t_{(5)} =0.46$). These results indicate that the starting population was not disturbed or altered by BDNF or olfactory deprivation. This was to be expected because the effects of increased BDNF under the CAMKIIα promoter were not experienced until the cells reached the bulb. The same concept applies with sensory deprivation in that the new cells do not experience the affect until present in the bulb.

The 28 day time point followed the established critical period for sensory dependent cell survival (Yamaguchi and Mori, 2005), and can reveal if BDNF over-expression partially inhibits the death that occurs due to deprivation during the sensitive phase. The current study yielded results in line with the previous study, with a decrease of ~30% in control GC populations and ~50% decrease in occluded populations between 12 and 28 days (Yamaguchi and Mori, 2005). Immunofluorescent staining indicated that ~98% of the surviving population was neuronal and cell counts were adjusted to reflect this. The results (Figure 9) suggest that the critical period was maintained in deprived TgBDNF animals as indicated by a significant decrease in numbers of surviving cells when comparing IBs and CBs in (IB=1290.5 ± 25.9, CB=1783.5 ± 34.4, $p<0.01$, $t_{(3)} =10.2$) or between IBs and controls (Contol= 1745.7 ± 109.3, $p<0.05$, $t_{(3)} =4.1$). There was also no significant difference in cell counts between WT versus Tg control animals (WT control = 2040.3 ± 127.6, $p=0.14$, $t_{(5)} =1.7$). These results indicate that over-expression of BDNF did not promote cell survival during the critical period of sensory deprivation.

The 56 day time point aimed to show the effects of BDNF on long term survival of these cells under conditions of continued sensory deprivation. Once again, immunofluorescent staining indicated that ~98% of the population was neuronal and cell

counts were adjusted to reflect this. Similar to the 28 day animals, Tg animals at 58 days showed a significant decrease in numbers of surviving cells when comparing IBs and CBs (Figure 9, IB=938.8 ± 49.4, CB=1232.8 ± 39.4, $p<0.05$, $t_{(3)} =4.2$) or between IBs and controls (Contol= 1124.7 ± 36.4, $p<0.05$, $t_{(5)} =3.0$) that was comparable to their WT counterparts (IB=881.4 ± 50.5, CB=1407.3 ± 152.4, $p<0.05$, $t_{(3)} =3.8$, Control= 1226.6 ± 110.5, $p<0.05$, $t_{(4)} =2.8$) . As before, there was no significant difference between cell counts in WT versus Tg control animals ($p=0.44$, $t_{(3)} =0.9$). This demonstrates that over-expression of BDNF under the CAMKIIα promoter does not affect long term survival of adult born GCs.

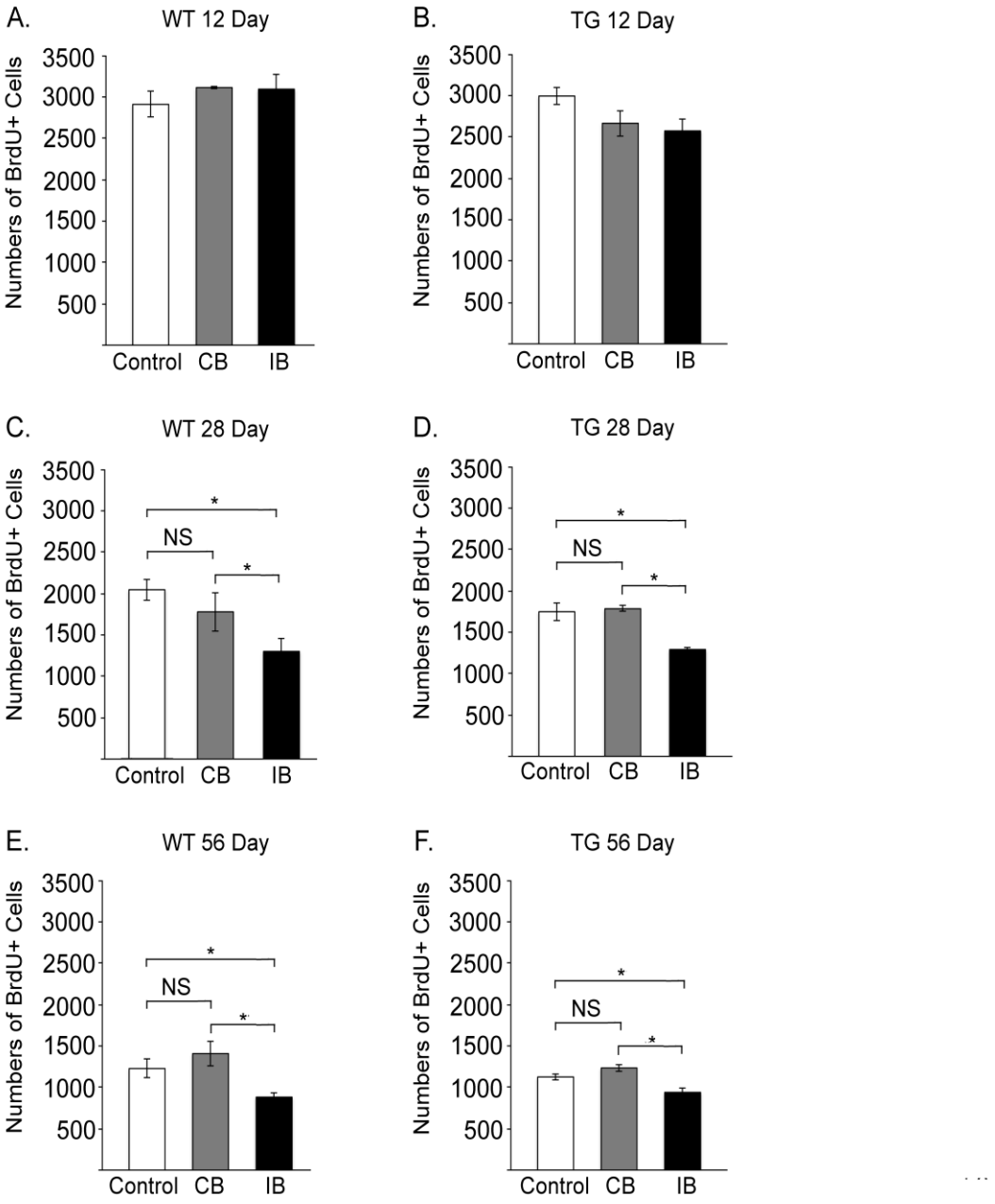

Figure 9: Effects of BDNF on populations of adult born granule cells
Bar graphs comparing numbers of BrdU+ neurons at each time point. (A, B) At 12 days post BrdU treatment WT and Tg animals showed no significant difference between control, contralateral (CB), or ipsilateral (IB) bulbs. (C, D,) At 28 days both genotypes showed significant decreases in numbers of BrdU+ neurons in IBs compared to CBs and controls. (E,F) At 56 days both genotypes showed significant decreases in numbers of BrdU+ neurons in IBs compared to CBs and controls. *P<0.05.

BDNF over-expression did not reduce programmed cell death

The 28 day time point was chosen because this is the endpoint of the critical period for sensory deprivation (Yamaguchi and Mori, 2005). The TUNEL reaction used a peroxidase staining technique for permanent labeling of cells undergoing DNA fragmentation, indicating apoptotic cell death (Figure 8e). Studies have shown that the number of TUNEL+ cells in the GCL significantly increases under sensory deprived conditions (Yamaguchi and Mori, 2005). Therefore, this technique could point to whether the increased expression of BDNF in vivo counters this affect by reducing cell death. To make this determination, the numbers of apoptotic cells in WT and Tg populations (IB, CB, and control) were compared. An unpaired, two tailed t-test showed that there was no significant difference in numbers of apoptotic cells between WT and Tg controls (Figure 10, WT=134.5 ± 10.3, Tg=146.5 ± 2.5, $p=0.34$, $t_{(3)} =1.1$). These results are comparable to previous findings (Smail et al., 2016). Paired, two tailed t-tests show that there was a significant increase in the number of apoptotic cells in the IB when compared to the CB and controls in both WT (Figure 10, IB=237.0 ± 19.5, CB=145.8 ± 23.4, $p<0.01$, $t_{(3)} =11.7$; versus Control $p<0.01$, $t_{(4)} =4.7$) and Tg (IB=272.5 ± 30.6, CB=124.0 ± 14.5, $p<0.05$, $t_{(3)} =3.7$; versus Control $p<0.05$, $t_{(3)} =4.1$) animals. While it cannot be determined if the apoptotic cells were adult born or not, the results suggest that over-expression of BDNF does not rescue GCs from apoptotic cell death under both normal conditions and olfactory deprivation.

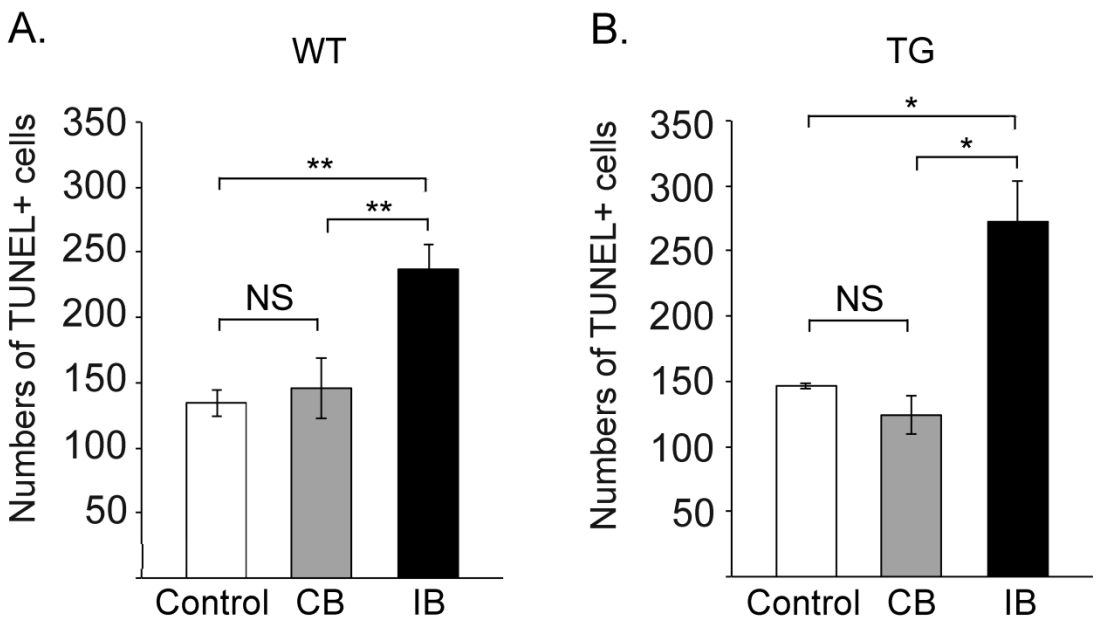

Figure 10: BDNF does not reduce programmed cell death of granule cells
Bar graphs comparing TUNEL+ cell counts in the GCL at 28 days. Bulbs ipsilateral to occlusion (IB) in WT and Tg mice showed significantly increased numbers of apoptotic cells in deprived bulbs when compared to contralateral (CB) and control bulbs. *P<0.05, **P<0.01.

Volume of the GCL in all deprived mice was decreased

At the 56 day time point, tissue sections from animals were collected in an ordered serial sequence and measured for GCL volume (mm^3). This time point was chosen because it represents the longest period of deprivation and therefore, is the time point in which the biggest change in volume could be observed following deprivation. The GCL shrinks over time with sensory deprivation due to loss of GCs (Cummings et al., 1997; Jin et al., 1996). The volume data (Figure 11) shows the extent to which the GCL atrophies in the deprived mice and whether the increased expression of BDNF counters this affect. Analysis using an unpaired, two tailed, t-test showed that there was

no significant difference in GCL volume of WT and Tg control animals (WT=1.97 ± 0.10, Tg=1.90 ± 0.07, p=0.58, $t_{(5)}$ =0.59), suggesting that BDNF does not have an overall effect in increasing GCL volume under normal conditions. Similar results were seen when comparing IBs and CBs in WT and Tg animals. IBs of WT animals showed a significant decrease in GCL volume when compared to the CB and control animals (IB=1.46 ± 0.13, CB=1.95 ± 0.13, p<0.05, $t_{(3)}$ =5.35; versus Control p<0.05, $t_{(5)}$ =3.01). In Tg animals, a significant decrease in GCL volume in IBs compared to CBs was seen as well (IB=1.19 ± 0.20, CB=1.68 ± 0.16, p<0.01, $t_{(3)}$ =6.6), suggesting that, under olfactory deprivation, Tg animals undergo GCL shrinkage due to cell loss that is similar to that of their WT counterparts despite the BDNF over-expression.

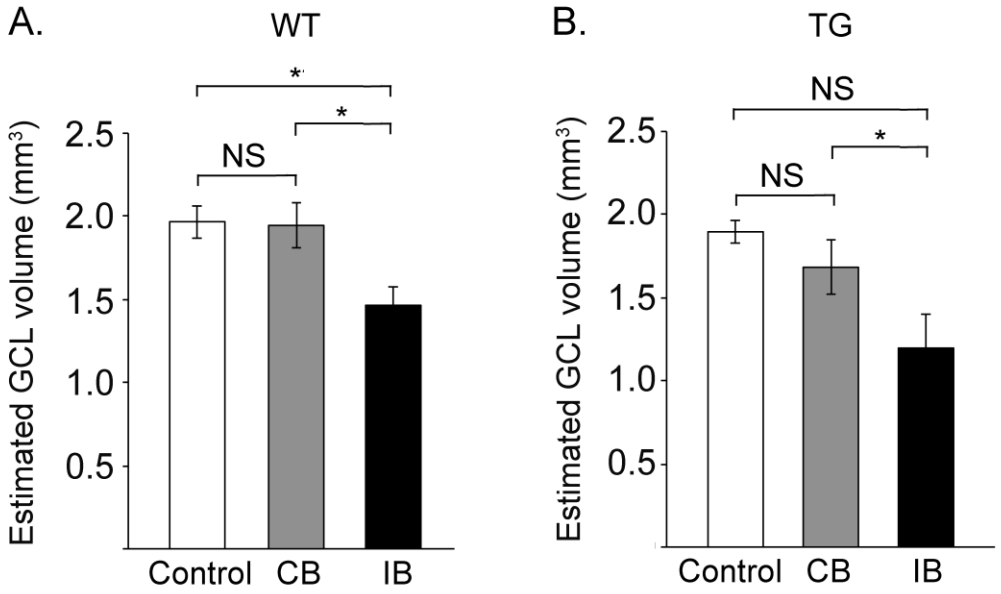

Figure 11: BDNF does not reduce GCL shrinkage due to unilateral naris occlusion.
Bar graphs comparing estimated GCL volumes (mm^3) between genotypes at 56 days. (A) Bulb ipsilateral to occlusion (IB) showed a significant decrease in GCL volume compared to bulbs contralateral to occlusion (CB) and controls in WT animals. (B) IBs also showed a significant decrease in GCL volume when compared to CBs in Tg mice. *P<0.05.

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IV. DISCUSSION

General Discussion

The results presented in the current study suggest that endogenous BDNF is not a survival factor for adult-born granule cells once they arrive in the olfactory bulb. The findings hold true whether this population of neurons is examined under normal conditions or under the effects of sensory deprivation. The transgenic mouse model that was utilized aimed to create an environment where endogenous BDNF was significantly increased in the olfactory bulb, where new neurons integrate. This is a brain area in which levels of BDNF are typically low in WT animals. Previous research showed that the levels of BDNF in this transgenic mouse model were, in fact, significantly increased when compared to WT counterparts (McDole et al., 2015). The current study confirms these results and also indicated that there was no significant change in bulb BDNF levels in sensory deprived, TG animals. These findings confirm that this mouse model was an appropriate one to use for the current study because the endogenous BDNF over expression was maintained under the CAMKIIα promoter despite a lack of sensory activity.

We also believe this to be a more appropriate model than that of previous studies, because the over-expression of BDNF occurred in the olfactory bulb. This is where the new neurons are exposed to BDNF under normal conditions and where they integrate into circuitry. Previous studies up-regulated expression of BDNF by injecting it into the SVZ,

a place where endogenous BDNF is not normally present (Benraiss et al., 2001; Bath et al., 2008; Galvao et al., 2008; Bath et al., 2012). At times, it is better to improve upon a system that already functions a particular way (introduction of extra BDNF into a system where it is normally present) rather than changing an entirely different system/structure. Furthermore, the multiple studies that employed this method to introduce excess BDNF have produced variable results. The SVZ is a region where stem cell proliferation occurs and neuroblast migration begins. It is not the place where these neurons are maturing, integrating, and surviving long term. Therefore, while these studies may show that the addition of BDNF into the SVZ has the potential to change the proliferating stem cell population, they fall short in being able to conclude whether or not BDNF is a survival factor in the environment the new neurons actually populate. Some studies have suggested an increase in integrating adult-born GCs at 3 weeks post SVZ injection of BDNF (Benraiss et al., 2001). However, this time point occurs before the end of the sensitive phase of programmed death of ~25% of new neurons, and did not take long term survival into account. In contrast, other studies showed that introduction of BDNF into the SVZ actually decreased survival of new neurons and that the conditional knockout of TrKB-FL had no effect on numbers of surviving GCs (Galvao et al., 2008; Bergami et al., 2013). Studies that suggested BDNF as a survival factor by using BDNF or TrkB knockout mice (non-conditional) are also not appropriate models because it is known that the lack of BDNF during early postnatal development is fatal in early postnatal life and causes developmental abnormalities (Linnarsson et al., 2000; Ernfors et al., 1994). It is difficult to draw conclusions based on a model that develops severe deficits early on and try to apply them to normal adult conditions. The over expression of

BDNF under the CAMKIIα promoter is an appropriate model for studying BDNF effects on adult-born GC incorporation and long term survival. This model is also uniquely useful because sensory deprivation does not affect the over-expression of the BDNF transgene and therefore, we are able to test whether up-regulating endogenous BDNF rescues neuronal survival after sensory deprivation.

The current study looked at the effects of BDNF over-expression in both "normal" and sensory deprived transgenic (Tg) mice. When comparing WT and Tg control mice, there was no observable difference between the two conditions for any of the experiments. There were no significant differences between WT and Tg BrdU+ cell counts, suggesting that excess BDNF had no effect on the starting population of cells that had migrated the bulb. This was to be expected because the cells were not exposed to the extra BDNF until they reached the bulb. This is also important because it means that the newly generated neurons had a "clean slate" at the beginning, and any further effects would be due to the increase in BDNF in the bulb itself. Findings from the 28 day time point suggest that excess BDNF does not increase the survival rate of adult-born GCs that are functionally integrating into the system. Twenty-eight days is the point at which new GCs are functionally incorporating into the OB circuitry and when a 25% decrease in numbers of new cells can be observed (Yamaguchi and Mori, 2005). Our results showed a similar decrease in new cell numbers in both WT and Tg animals suggesting that the over-expression of BDNF is not enhancing their survival. Results from TUNEL staining also revealed a statistically similar amount of cell death between genotypes. Similarly, this over-expression did not promote increased long term survival of adult born GC as indicated by the 56 day time point. The same steady decrease in the new cell population

from 28 to 56 days that has been previously reported was seen in both WT and Tg animals. These results are substantiated by the effects on GCL volume. Together with the BrdU cell counts, these findings show that similar cell survival was accompanied by similar levels of cell death in both genotypes under normal conditions. This is important because there is a significant and steady turnover rate of GCs, and these findings demonstrate that excess BDNF affected this rate in neither a positive or negative way. Comparisons between control WT and Tg animals mirrored the results of previous findings in this lab (Smail et al., 2016).

Although over-expression of BDNF did not affect the survival of adult-born granule cells in control animals, we sought to test whether the excess BDNF had an affect under circumstances that normally reduce cell survival. Unilateral naris occlusion is an effective model that has been utilized in many studies over the years. Naris occlusion deprives the bulb of odor stimulation (sensory activity), and serves as a non-invasive way of shutting down normal neural activity in the bulb. Adult-born GCs then must integrate and survive in an environment that is not favorable. These results may extend to similar limitations in the survival of new neurons in the presence of other unfavorable conditions (i.e. neurodegeneration due to disease or injury).

Results from deprived animals were similar to results from the control animals. Therefore, over-expression of BDNF in the olfactory bulb did not reduce effects of sensory deprivation due to unilateral naris occlusion. These findings were consistent throughout all time points of the study. The IBs at the 12 day time point maintained statistically similar cell counts to that of the CB and control bulbs in both the WT and Tg animals. Again, this was to be expected because at 12 days the cells are just starting to

enter the bulb and have not experienced the effects of both the BDNF and the sensory deprivation.

The 28 day time point was a crucial part of the current study. As previous studies suggest, the critical period for sensory-dependent survival of adult-born GCs ends at approximately 28 days (Yamaguchi and Mori, 2005). Therefore, this is the best time point to observe potential survival effects under deprived conditions. The results show that over-expression of BDNF has no affect on the survival of adult born GCs during the sensitive period. New granule cell populations in both WT and Tg animals experienced the same 50% decrease in deprived bulbs that has been previously observed when compared to normal bulbs. In agreement with this finding, there was also a significant increase in TUNEL+ cells in the IBs of both deprived WT and Tg animals, indicating increased cell death. One limitation to TUNEL staining however is that there is no way to determine if the populations labeled were new adult-born GCs or older GCs. A previous study by Bastien-Dionne (2009) revealed that sensory deprivation reduces both newborn GCs and GCs that were present prior to naris occlusion. This along with the BrdU results of the current study leads us to believe that the increase in TUNEL+ cells likely occurred in both new and older populations of GCs. Overall, the findings at this time point demonstrate that increased endogenous BDNF does not rescue adult-born neurons during the critical period of sensory deprivation.

At 56 days post occlusion (the latest time point), results from the Tg animals mirrored that of the WT animals as well. These results revealed that the increase in BDNF did not aid in the long term survival of adult born GCs in a sensory deprived environment. This was observed through the analysis of BrdU cell counts as well as GCL

volume. Cell counts in Tg mice showed the same steady decrease that was seen in WT mice following the end of the critical period (Yamaguchi and Mori, 2005). Moreover, the deprived Tg animals displayed the same loss of GCL volume to the IB that was observed in WT animals and that has been reported in previous studies (Jin et al., 1996). GCs that survive past the initial critical period (under normal conditions and under sensory deprivation) typically survive long term; although there is still a small, steady decrease that occurs gradually. If BDNF functioned as a long term survival factor, we would expect to see more new cells at this time point given that this is the point where continuing survival appears stable. However, we found this is not the case, which further confirms that BDNF does not support survival of adult-born GCs in the absence of sensory stimulation.

There are some considerations worthy of mention; the biggest being the activity-dependent transport and release of BDNF. Following synthesis, BDNF (pro- and mature) is packaged into the trans-Golgi network (TGN). From here it can have two fates. Small vesicles containing BDNF can bud off from the TGN and be released constitutively, or BDNF can be stored in dense-core granules and await release via activity-dependent pathways (Lessmann and Brigadski, 2009; Hartmann et al., 2012). Constitutive release reflects the constant gradual secretion of BDNF while activity-dependent secretion occurs rapidly with depolarization at the synapse. Previous studies have revealed that the way in which BDNF is released may be as important as concentration when looking at neuronal function (Matsuda et al., 2009; Hartmann et al., 2012). Therefore, it is possible that although the ELISA measurements in the current study revealed that protein concentration remained elevated in naris-occluded Tg mice, these animals were not fully

utilizing the activity-dependent secretion mechanism due to low sensory drive as a result of the deprivation. Since constitutive release is slow and gradual, it is possible that there is a plateau in terms of how much BDNF can be released constitutively from a cell despite the increase in protein synthesis. If this is the case, the cells would need to rely on activity in order to release the extra BDNF being produced. For this reason, it is possible that the negative results of the current study are not due to the BDNF's inability to rescue cells, but rather because of the cells limited ability to release the BDNF without adequate sensory stimulation. In other brain regions deprived of sensory stimulation, particularly in the visual cortex, excess BDNF *does* prevent impaired development (Gianfranceschi et al., 2003). However, this area does not undergo cell death, so neuronal survival effects are not testable.

It is also possible that there is a down regulation of TrkB expression with deprivation, and we have not measured this possible effect. If TrkB expression was activity dependent, there is a possibility that the negative results from the current study were due to greater competition for TrkB binding. However, this is most likely not the case. A recent study has shown that conditional knockout of TrkB *altogether* does not affect survival of adult-born GCs, under normal conditions (Bergami et al., 2013). Moreover, another study showed while dark rearing (total lack of visual stimuli) decreased phosphorylated TrkB, it does not decrease TrkB receptor expression (Viegi et al., 2002). The study then infused the area with BDNF and the levels of phosphorylated TrkB returned to normal. Since there was increased BDNF expression in our transgenic mouse model, the levels of TrkB and phosphorylated TrkB were most likely maintained in the odor-deprived TG mice.

The results from the current study indicate that BDNF is not a survival factor for adult-born GCs in the rodent olfactory bulb under both normal and sensory deprived conditions. However, this does not mean that the over-expression of BDNF in this system has no effect at all. Previous work in our laboratory has showed that over-expression of BDNF increases GC spine density, while the conditional TrkB knockout study demonstrated reduced spine density on these cells (Bergami et al., 2013; McDole et al., 2015). These results suggest that BDNF plays a key role in GC spine development, synaptic stabilization, and plasticity. Since it is also known that sensory deprivation reduces spine density in adult born GCs (Dahlen et al., 2011), it is conceivable that over-expression of BDNF in the olfactory bulb may counteract some of the morphological deficits seen with sensory deprivation, rather than effecting the survival of the cells.

Conclusion

Based on the findings in the current study, increasing endogenous BDNF expression in the olfactory bulb does not increase survival of adult born GCs when they are deprived of sensory stimulation. Through the use of a transgenic mouse model which over-expresses BDNF and our histological findings, we observed that increased BDNF did not counteract neuronal death due to sensory deprivation.. These findings are in line with Galvao et al. (2008), and with Bergami et al. (2013). However, it is possible that future studies examining activity-dependent secretion of BDNF may further test BDNF's potential as a promoter of dendritic spine development and maturation in this system. Evidence has shown that both olfactory deprivation and BDNF signaling play a role in shaping the dendritic morphology of GCs (Dahlen et al., 2011; Bergami et al., 2013; McDole et al., 2015). It would be of interest to determine BDNF's potential role in

counteracting morphological deficits of sensory deprived GCs. BDNF may drive dendritic development, spine formation, and maintenance in adult-born GCs, rather than control their survival. This would indicate an important role for BDNF in regulating developing connectivity of new neurons in pre-existing neural networks.

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