Behavioral consequences of increasing adult hippocampal neurogenesis

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Submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
under the Executive Committee
of the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY

2014

ABSTRACT

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The hippocampus is a brain structure involved in memory as well as anxiety and depression-related behavior. One unique property of the hippocampus is that adult neurogenesis occurs in this region. Rodent studies in which adult hippocampal neurogenesis is ablated have shown a role for this process in the cognitive domain, specifically in pattern separation tasks, as well as in mediating the behavioral effects of antidepressants. These studies have furnished the intriguing hypothesis that increasing adult hippocampal neurogenesis may improve these functions and therefore serve as a target for novel treatments for cognitive impairments as well as depression and anxiety disorders. Here, we use both genetic and pharmacological models to increase adult neurogenesis in mice. Under baseline conditions, we find that increasing adult hippocampal neurogenesis is sufficient to improve performance in a fear-based pattern separation task, but has no effect on exploratory, anxiety or depression-related behavior. In mice exposed to voluntary exercise, increasing adult hippocampal neurogenesis increases exploration, without affecting anxiety or depression-related behavior. Finally, in mice treated with chronic corticosterone, a model of anxiety and depression, increasing adult hippocampal neurogenesis is sufficient to prevent the behavioral effect of CORT on anxiety and depression-related behavior. Here, we therefore describe dissociations between the effects of increasing adult hippocampal neurogenesis under baseline, voluntary exercise and chronic stress conditions. Together, our results suggest that increasing adult hippocampal neurogenesis has therapeutic potential for both cognitive, and anxiety and depression-related disorders.

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Acknowledgements

First, I would like to thank my mentor, Dr. René Hen, for the guidance provided during my time in the lab. Our discussions about my projects were always immensely helpful, educational, and fun! I am enormously grateful to have had the opportunity to complete my thesis in his lab.

I would like to thank the former and current postdoctoral fellows and graduate students in the Hen lab for their assistance. Amar Sahay mentored me on most of the projects described in this thesis, and taught me so much regarding both experimental techniques and how to be a scientist. I am extraordinarily grateful to have had the opportunity to work with and learn from him. Thank you to Zoe Donaldson for providing extensive guidance in generating the Decorin:CreERT2 mouse line. I greatly benefited from learning molecular biology techniques from her. I am thankful to Susanne Ahmari and Mazen Kheirbek for teaching me rodent surgery and optogenetic techniques, and for providing guidance on these experiments. Thank you also to Neria Douglass for her help.

Thank you to Melody Wu, Katherine Nautiyal, Nesha Burghardt, and Christine Denny for immense amounts of guidance throughout the years. Thank you also to Ben Samuels, Liam Drew, Victor Mari-Luna, Matthew Wright, Bradley Miller, Christoph Anacker, Kristen Klemenhagen, Clay Lacefield, Alyssa Pichinni, Jesse Richardson-Jones, Kim Scobie, Lindsay Tannenholz, Rebecca Brachman and Jessica Jimenez. I have learned from and enjoyed working with every one of you. Thank you to Navieta Ramasami for your assistance and enduring patience. Thank you to K. Keener, Max Lauring, Nicola Chan and Camille Price for volunteering to work with me.

I would like to thank the directors of the Neurobiology and Behavior Program: Carol Mason, Darcy Kelley and Ken Miller. Thank you as well to Gilbert Di Paolo, Brian McCabe, and their laboratories for guidance while I rotated in these labs during my first year of graduate school. I would also like to thank my previous advisors and mentors: Flavia Awad, Dr. Ponzy Lu and Dr. Edward Cooper, who encouraged me to pursue scientific research.

Thank you to my thesis committee: Holly Moore, Christoph Kellendonk, Mark Ansorge, Jay Gingrich and Tracey Shors, for helpful insight, guidance and discussions. Lastly, I would like to thank the animal care facility at New York State Psychiatric Institute, especially the Rodent Neurobehavioral Analysis Core, where much of the work in this thesis was conducted.

Chapter 1: Introduction

1.1 The hippocampus

The hippocampus is a large, bilateral brain region that has been implicated in playing a role in both memory and mood-related behavior. The hippocampus is made up of subregions with distinct connectivity and functions, which are highly homologous in humans and mice. Therefore, extensive experimentation has been conducted in mice to understand the structure and function of this brain region, with presumed implications for memory and mood-related function in humans.

1.1.1 Anatomy

The hippocampus is comprised of three main connected subregions: the dentate gyrus, area CA3 and area CA1, as originally described by Lorente de No (Lorente de No 1934). Additionally, the hippocampal complex includes two cortical regions located posterior to the hippocampal proper: the entorhinal cortex, which provides the main glutamatergic input to the hippocampus, and the subiculum, which along with CA1, provides the main hippocampal output. In addition to glutamatergic input from the entorhinal cortex, the hippocampus also receives excitatory input from the mammillary bodies (Wyss et al. 1979, Kiss et al. 2000) as well as serotonergic input from the raphe nucleus (Conrad et al. 1974, Moore and Halaris 1975), cholinergic input from the medial septum (Mosko et al. 1973, Amaral and Kurz 1985), noradrenergic input from the locus coeruleus (Swanson and Hartman 1975), and dopaminergic input from the ventral tegmental area (Gasbarri et al. 1997).

Within the hippocampus, the main pathway of synaptic connectivity is described by the trisynaptic circuit, which consists of projections from entorhinal cortex to dentate gyrus (perforant path), dentate gyrus to CA3 (mossy fibers) and CA3 to CA1 (Schaffer collaterals) as depicted in Figure 1.1. However, there are additional synaptic connections between these regions, including projections from entorhinal cortex to CA3 and CA1, as well as numerous excitatory and inhibitory interneurons that project within and between subregions (not shown in diagram). The hippocampus extends from an antero-dorsal pole to a postero-ventral pole, referred to as the longitudinal axis. Throughout the longitudinal axis, circuitry within the hippocampus is maintained such that the principal cells of each hippocampal subregion (dentate gyrus, CA3, CA1) project to cells located within a similar plane of the longitudinal axis.

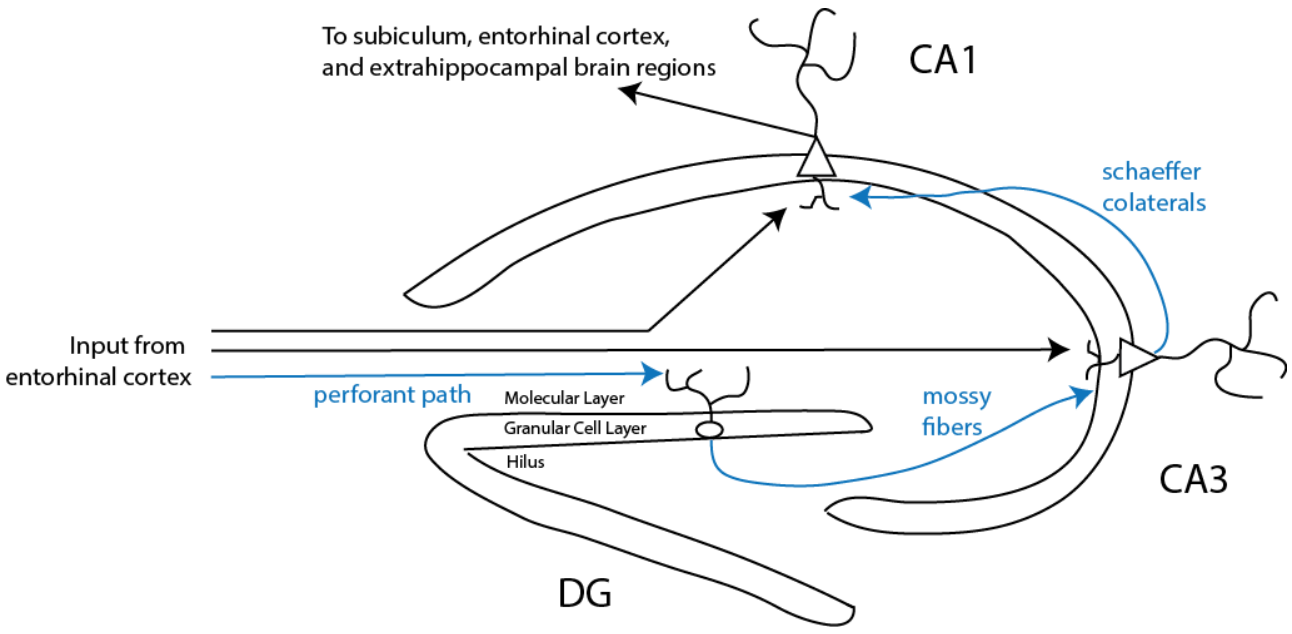

Figure 1.1 Diagram of the hippocampus
Hippocampal circuitry consists of the trisynaptic circuit (shown in blue), additional projections from the entorhinal cortex to areas CA3 and CA1 (shown in black), as well as many classes of interneurons that project within and between subregions (not shown). Dentate gyrus (DG).

The dentate gyrus is easily recognized by the densely packed cell bodies of granule cells, the principle cells of this region, which are found in what is referred to as the granule cell layer, organized into two blades that make up a sideways v-shaped structure (Figure 1.1). Dentate granule cells project to CA3 via axons referred to as mossy fibers, due to the many varicosities found along these axons that give them a "mossy" appearance. The mossy fibers form a powerful "detonator"-like synapse onto CA3 pyramidal cells, where bursting from one single mossy fiber has been found to be sufficient to induce firing in a downstream CA3 pyramidal cell (Henze et al. 2002). Between the two blades of the dentate gyrus is the hilus, a polymorphic region filled with interneurons, as well as granule cell mossy fiber axons. Between the hilus and the granule cell layer is a thin layer referred to as the subgranular zone (SGZ), a vascular niche where stem cells are located that produce adult-born granule cells.

1.1.2 Function

Through various studies in both rodents and humans, the hippocampus has been shown to play a role in both memory-related tasks as well as anxiety and mood-related behavior.

Patient and animal lesion studies initially implicated the hippocampus as an important brain region for memory. In the famous case study of H.M., resection of the temporal lobes, which includes the hippocampus, led to severe anterograde amnesia, an inability to create new explicit memories (Scoville and Milner 1957). Hippocampal lesions have also been shown to impact memory in rodents, impairing performance in fear conditioning tasks, in which a mouse learns to associate a shock with a context (Phillips and LeDoux 1992), and in spatial memory tasks such as the Morris water maze, where a mouse uses visual cues to learn the location of a hidden platform in an arena filled with water (Morris et al. 1982).

There has long been interest in understanding how the hippocampus encodes memories, and the connectivity of hippocampal subregions has been used to develop theoretical hypotheses underlying memory processes. As relevant to this thesis, there are two distinct and unique characteristics of hippocampal circuitry that have generated hypotheses about memory. The first is the dense recurrent collateral projections between CA3 pyramidal cells, forming an auto associational network (Swanson et al. 1981, Rolls 2013). This type of connectivity is thought to connect cells representing different aspects of a given experience, underlying the process of pattern completion, through which a full memory or experience can be remembered when only partial cues are presented (Marr 1971, O'Reilly and McClelland 1994, Rolls 1996, McHugh et al. 2007).

A second characteristic of hippocampal circuitry that is theorized to be important for memory, are the mossy fiber inputs from dentate granule cells to CA3 pyramidal cells. These inputs are relatively sparse (Amaral et al. 1990), and granule cells are known to have relatively low levels of activity (Jung and McNaughton 1993, Chawla et al. 2005), therefore allowing a large coding space, through which there are many possible patterns of activation. This is thought to orthogonalize the activity from similar inputs, thus underlying pattern separation, the process through which similar experiences are distinguished (O'Reilly and McClelland 1994). Supporting this hypothesis, the activity of dentate granule cells has been shown to be sensitive to slight changes in environment (Leutgeb et al. 2007). Additionally, the large mossy fiber terminal boutons provide a powerful input (Henze et al. 2002), enabling the relatively sparse input from the dentate gyrus to significantly influence CA3 activity. This input is therefore thought to override activity in recurrent collaterals, allowing for the formation of new associations between CA3 pyramidal cells, which may underlie new memory formation (Treves and Rolls 1992).

The dentate gyrus has been implicated as playing a role in pattern separation based on animal behavioral studies thought to require pattern separation. Rodents with dentate gyrus lesions display impaired behavior in a matching to place task, in which animals must discrimination between subtle differences in object placement (Gilbert et al. 2001, Hunsaker et al. 2008). Pattern separation has also been tested using contextual fear discrimination learning, where animals are exposed to two similar contexts, only one in which they receive a shock (Wehner and Radcliffe 2004), and it has been shown that mice lacking the NR1 subunit of the NMDA receptor specifically in dentate granule cells are impaired in contextual discrimination in this type of task (McHugh et al. 2007). In humans, when people are shown two pictures of similar items and need to determine whether the pictures are the same or different, increased activation in functional magnetic resonance imaging (fMRI) experiments is seen in the dentate gyrus and CA3 (Bakker et al. 2008, Lacy et al. 2011), suggesting that in humans these brain regions may also be involved in pattern separation and completion. Together, rodent and human studies have implicated a role for the hippocampus in memory, yet the precise hippocampal mechanisms underlying processes such as pattern separation and completion are not fully understood.

The hippocampus has also been shown to play a role in regulating mood. Human imaging studies have reported decreased hippocampal volume in patients with a history of depression (Sheline et al. 1996, Videbech and Ravnkilde 2004), and increased hippocampal volume following antidepressant treatment (Frodl et al. 2008), suggesting that hippocampal processes may be involved in depression and antidepressant action.

Animal studies have further implicated the hippocampus as an important brain structure in mediating the effects of antidepressants, as several changes in the hippocampus are observed

after antidepressant treatment, some of which have been shown to be necessary or sufficient for the effects of antidepressants on mood-related behavior. For example, in rodents, adult hippocampal neurogenesis has been shown to be necessary for some of the behavioral effects of antidepressants (Santarelli et al. 2003), while overexpression of CREB or BDNF in the dentate gyrus is sufficient for antidepressant-like effects on behavior (Chen et al. 2001, Shirayama et al. 2002). The hippocampus is therefore a key structure for modulating mood.

1.1.3 Dorsal and ventral hippocampal subregions

Accumulating evidence suggests that the hippocampus can be split into two compartments along the longitudinal axis, referred to as dorsal and ventral subregions, which vary in anatomical connectivity, genetic expression and function (Fanselow and Dong 2010).

Anatomical differences along the longitudinal axis have been described in detail (Fanselow and Dong 2010). Although the internal synaptic connectivity within the hippocampus is preserved throughout the longitudinal axis, input and output to the hippocampus varies. The distinction between connections of the dorsal and ventral hippocampus was initially described in extensive tracing studies by Swanson and Cowan, showing that efferent projections vary along the longitudinal axis (Swanson and Cowan 1977). Interestingly, projections from the dorsal hippocampus project to regions thought to be involved in navigation and locomotion, such as the mammillary nuclei and anterior thalamus (Taube 2007), as well as to the ventral tegmental area through a relay in the lateral septum (Swanson and Kalivas 2000, Luo et al. 2011). On the other hand, the ventral hippocampus projects to regions thought to be involved in emotion, motivated behavior and regulation of the neuroendocrine systems, such as the amygdala, bed nucleus of the stria terminalis (BNST) and hypothalamus (Fanselow and Dong 2010). Hippocampal *input* also

varies along the longitudinal axis. Entorhinal projections to the dorsal and ventral hippocampus originate from different regions of the entorhinal cortex (Dolorfo and Amaral 1998), which receive input from different cortical regions (Burwell and Amaral 1998). Additionally, serotonergic input is more dense in the ventral dentate gyrus (Gage and Thompson 1980), and serotonin receptors have different expression levels along this axis (Tanaka et al. 2012).

Genomic studies have shown that dozens of genes have varied expression along the hippocampal longitudinal axis (Leonardo et al. 2006) (Allen Brain Atlas). The dentate gyrus, CA3 and CA1 subregions can each be further divided along this axis into subregions with different expression profiles based on this data (Thompson et al. 2008, Dong et al. 2009).

Initial indications that dorsal and ventral hippocampal regions have distinct functions came from lesion studies, which showed that lesions specifically of the dorsal hippocampus impair performance on cognitive-related tasks including the Morris water maze, fear conditioning and the radial arm maze (Moser et al. 1995, Pothuizen et al. 2004), while lesions specifically of the ventral hippocampus decrease anxiety-like behavior, in tests such as the elevated plus maze (Kjelstrup et al. 2002, McHugh et al. 2004). Within the dentate gyrus, this distinction has been further characterized using optogenetics, which has shown that disruption of activity of dorsal dentate granule cells impairs contextual fear conditioning, while activation of ventral dentate granule cells decreases anxiety (Kheirbek et al. 2013). Together, anatomical, genetic expression and functional studies show that dorsal and ventral hippocampal regions have distinct properties.

1.2 Adult hippocampal neurogenesis

An interesting feature of the hippocampus is the presence of neural stem cells that continue to produce new neurons throughout adulthood. This process of neurogenesis has been observed in humans, and in rodents, it has been shown to play an important role in mediating hippocampal function.

Although neurogenesis was long thought to only occur during development, we now know that it continues in specialized regions of the adult brain. In the 1960s, Altman and Das first described the presence of neurogenesis in the adult hippocampus of the rat (Altman and Das 1965), and many subsequent studies have characterized two neurogenic niches in the adult rodent brain: the subgranular zone in the dentate gyrus, and the subventricular zone, where adult-born cells are produced that migrate to the olfactory bulb (Altman 1969, Lois and Alvarez-Buylla 1994). In the 1980s, this finding was extended to the presence of adult neurogenesis in the female canary (Goldman and Nottebohm 1983), and to many regions in other fish, amphibians, reptiles and birds (Barker et al. 2011).

In the 1990s, the first report of neurogenesis in the adult human hippocampus was reported in cancer patients who were injected with bromodeoxyuridine (BrdU), a thymidine analog that becomes incorporated into the DNA of dividing cells at the time of injection (Eriksson et al. 1998). This technique is also widely used in rodent studies. BrdU becomes incorporated not only into cells that are dividing at the time of injection, but also into any progeny from these cells, allowing assessment of levels of neurogenesis from the time of injection until death.

Adult neurogenesis in humans has also been assessed using Carbon-14 levels in brain tissue samples. Since the environmental levels of Carbon-14 have varied over the last several

decades due to nuclear bomb testing in the 1950s and 1960s, comparison of Carbon-14 levels in brain tissue with the expected levels that would be found in cells born either at the time of an individual's birth or during an individual's adult life, indicates whether adult neurogenesis has occurred (Spalding et al. 2005). These studies have provided additional evidence for adult-born neurons in the human hippocampus (Spalding et al. 2013). Unexpectedly, a recent study using this method also reported evidence of adult-born neurons in the striatum (Ernst et al. 2014). It has therefore been suggested that in humans, adult-born cells may migrate from the SVZ to the striatum rather than to the olfactory bulb, as has been found in rodents, since cells have not been found in the human olfactory bulb that were born after the 1st year of life (Bergmann et al. 2012).

1.2.1 Process of adult hippocampal neurogenesis

Since these initial discoveries, much work has been conducted to elucidate the detailed process of adult hippocampal neurogenesis. Adult-born neurons in the hippocampus are generated from radial glia-like stem cells located in the subgranular zone (SGZ), a thin region inside the inner granule cell layer, which is characterized by dense vasculature, forming a niche for the production of new neurons (Palmer et al. 2000). During the process of adult hippocampal neurogenesis, stem cells in the SGZ divide to primarily produce cells that mature into neurons, however astrocytes, and possibly oligodendrocytes, are also produced (Bonaguidi et al. 2011, Dranovsky et al. 2011, Chetty et al. 2014).

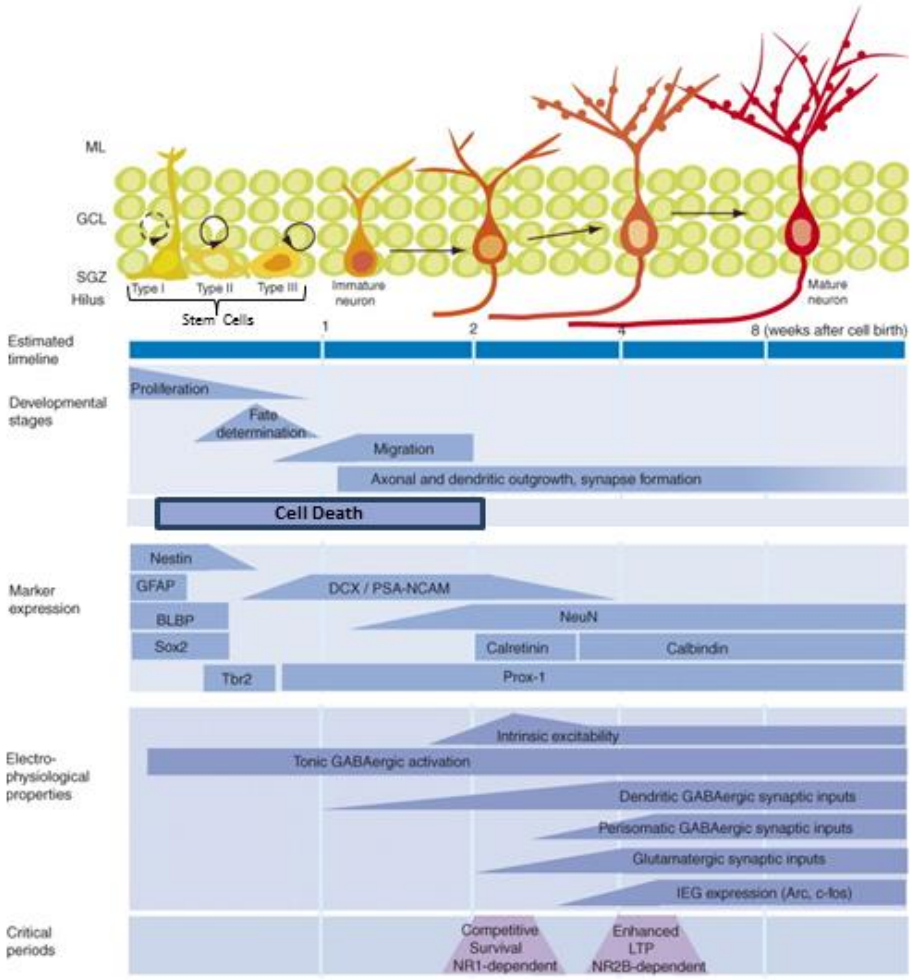

Figure 1.2 Adult hippocampal neurogenesis schematic
Schematic of adult hippocampal neurogenesis and properties of maturing adult-born cells. Adapted from (Duan et al. 2008).

Stem cells located in the adult SGZ have been characterized into three types based on morphology and genetic expression. The first, 'Type I' stem cells, also referred to as radial glia-like cells, are characterized by an apical dendrite that courses through the granule cell layer, while Type II and Type III cells have only short processes (Seri et al. 2001, Duan et al. 2008). As can be seen in Figure 1.2, different cell types can be defined based on genetic expression profiles. Type I stem cells express glial fibrillary acidic protein (GFAP), an intermediate filament protein that is also expressed by astrocytes; Type I and Type II cells both express nestin, another

intermediate filament protein expressed transiently during development; and Type III cells express neither of these proteins (Fukuda et al. 2003).

The promoters of these genes have been used to make transgenic mouse lines, which have been used to characterize and manipulate adult hippocampal neurogenesis. These studies have shown that Type I stem cells are generally quiescent, slowly dividing cells, while Type II cells, also referred to as transient amplifying cells, divide more rapidly (Filippov et al. 2003, Kronenberg et al. 2003).

An additional complexity to the process of adult hippocampal neurogenesis is that during maturation, 60-80% of adult-born neurons undergo cell death between 1 day and 2 weeks of age (Cameron et al. 1993, Sierra et al. 2010). Adult-born neurons that survive this period continue to mature by extending dendrites into the molecular layer (ML in Figure 1.2) of the dentate gyrus, and an axon through the hilus to CA3. Immature adult-born neurons can be identified by expression of doublecortin (DCX), a microtubule binding protein involved in migration of developing neurons, which is expressed in granule cells until four weeks of age (Brown et al. 2003). By four weeks of age, adult-born neurons express mature neuronal markers (van Praag et al. 2002).

Adult-born hippocampal neurons have unique electrophysiological properties as they develop into mature granule cells. During the maturation process, young neurons display increased excitability (Wang et al. 2000, Schmidt-Hieber et al. 2004, Esposito et al. 2005), which is thought to affect the dentate gyrus and hippocampal signaling in a unique manner (Deng et al. 2010). As opposed to fully mature granule cells, developing adult-born granule cells express the chloride importer NKCC1 (Ge et al. 2006), and the NMDA receptor subunit NR2B (Ge et al. 2007), which lead to a depolarized resting membrane potential, high levels of intracellular

chloride and high input resistance, which produce a hyperexcitable state (Ge et al. 2006, Mongiat et al. 2009). When adult-born neurons are four to six weeks old, they exhibit NR2B-dependent enhanced excitability, which is thought to confer a critical period of enhanced plasticity (Ge et al. 2007). After this stage, adult-born cells appear to be electrophysiologically equivalent to granule cells generated during development (Laplagne et al. 2006).

Adult-born granule cells have been estimated to comprise up to 10% of the granule cells in the mouse dentate gyrus (Imayoshi et al. 2008). Activity of adult-born cells can be detected in slice recordings by inducing a specific form of long-term potentiation (LTP). Most granule cells are normally inhibited, therefore LTP is typically assessed in the presence of gamma-aminobutyric acid (GABA) receptor blockers (Wigstrom and Gustafsson 1983). However, in a slice bathed in artificial cerebrospinal fluid (ACSF) without GABA receptor blockers, a smaller LTP can be detected, which has been shown to be dependent on adult-born granule cells, as it is lost upon ablation of adult neurogenesis by x or gamma-irradiation (Snyder et al. 2001, Saxe et al. 2006). This form of LTP (referred to as ACSF-LTP) is enhanced by manipulations that increase neurogenesis, such as antidepressant treatment (Wang et al. 2008). The role of adult-born neurons in ACSF LTP provided the initial evidence that these cells have unique firing properties that may allow them to serve a unique function.

In addition to the hyperexcitable state of adult-born neurons, mounting evidence shows that adult hippocampal neurogenesis decreases overall activity in the dentate gyrus. Ablation of neurogenesis has been shown to increase perforant path evoked responses (Lacefield et al. 2012), and calcium imaging has shown a similar effect, while also showing that increased levels of adult hippocampal neurogenesis decrease overall activity in the dentate gyrus (Ikrar et al. 2013). Adult-born neurons have been hypothesized to decrease activity in the dentate gyrus through

disinhibition of mature granule cells, via connections through inhibitory interneurons (Kheirbek et al. 2012, Lacefield et al. 2012, Song et al. 2012), and our lab has recently found that optogenetically stimulating adult-born neurons inhibits mature granule cells in a slice preparation (Hen Lab, unpublished).

1.2.2 Functions of adult hippocampal neurogenesis

Initial studies characterizing the process of adult hippocampal neurogenesis showed that levels of neurogenesis are increased in mice exposed to learning tasks (Gould et al. 1999), and decreased by treatment with the stress hormone corticosterone (Cameron and Gould 1994), leading to the hypotheses that adult neurogenesis might be involved in memory and mood. Just as the hippocampus as a whole plays a role in both cognitive and mood-related functions, adult hippocampal neurogenesis also appears to be involved in both of these domains. In order to directly assess the role of adult hippocampal neurogenesis, techniques were developed to ablate adult-born cells, including focal x-irradiation and transgenic mouse lines to kill adult-born neurons (Santarelli et al. 2003, Garcia et al. 2004).

The contribution of adult hippocampal neurogenesis to learning and memory

Ablation of adult hippocampal neurogenesis has implicated this process in various hippocampal-dependent memory paradigms. Ablated adult hippocampal neurogenesis has been shown to impair trace eye blink and trace fear conditioning (Shors et al. 2001, Shors et al. 2002), as well as contextual fear conditioning in some studies (Saxe et al. 2006, Winocur et al. 2006, Imayoshi et al. 2008), but not in others (Shors et al. 2002, Clark et al. 2008). A study from the

Hen lab has suggested that it is specifically in difficult fear conditioning paradigms with little training where adult hippocampal neurogenesis affects learning (Drew et al. 2010).

Other studies have suggested that four to six week old neurons uniquely contribute to performance in these learning tasks. Along with their distinct electrophysiological properties, four to six week old neurons are preferentially activated and incorporated into circuits for spatial memory (Kee et al. 2007), and uniquely contribute to contextual fear conditioning (Denny et al. 2012).

Adult neurogenesis has also been shown to contribute to pattern separation-based tasks. Since the dentate gyrus is thought to act as a pattern separator by reducing overlap between inputs to CA3, the addition of new neurons is thought to increase this capacity in two possible ways. One way is through the constant addition of new units that have increased excitability and plasticity during the critical period of their maturation (Aimone et al. 2010, Sahay et al. 2011). The second way is through decreasing overall activity levels of the dentate gyrus (Lacefield et al. 2012, Ikrar et al. 2013). Since the ability of the dentate gyrus to act as a pattern separator is partially dependent on sparse activity, decreasing overall activity levels would make this region even more sparsely activated, thereby enhancing pattern separation.

Experimentally, ablation of adult neurogenesis in rodents has been shown to impair performance in pattern separation-based tasks, in both spatial and contextual learning paradigms (Clelland et al. 2009, Nakashiba et al. 2012, Tronel et al. 2012). Interestingly, transgenic mice with NR2B deleted specifically from adult born neurons do not display neurogenesis-dependent ACSF-LTP (Snyder et al. 2001, Ge et al. 2007), and are impaired in a pattern separation-based task (Kheirbek et al. 2012), suggesting that NR2B-dependent enhanced plasticity of adult-born cells is required for their role in pattern separation. Additionally, voluntary exercise, which

increases neurogenesis, has been shown to improve performance in a spatial pattern separation task (Creer et al. 2010). Together, these studies suggest that changes in levels of adult hippocampal neurogenesis impact the performance of rodents in tasks requiring pattern separation, supporting a role for adult hippocampal neurogenesis in this process.

While the majority of initial studies supported a positive role for adult hippocampal neurogenesis in improving learning and memory, other studies have suggested that adult hippocampal neurogenesis may not be beneficial for all types of memory. Saxe et al. found that ablation of adult neurogenesis *enhanced* performance in a radial arm maze task, specifically in paradigms that require discrimination between similar cues presented closely in time after a temporal delay, a task in which mice may need to disregard conflicting information from previous trials (Saxe et al. 2007). A more recent study has further supported the hypothesis that adult hippocampal neurogenesis may disrupt the stability of previously formed memories, providing evidence that increasing neurogenesis impairs performance in a remote contextual fear conditioning task (Akers et al. 2014). Adult hippocampal neurogenesis might therefore bidirectionally modulate different memory processes, in potentially beneficial or harmful manners.

The contribution of adult hippocampal neurogenesis to mood-related behavior

Evidence for a role of adult hippocampal neurogenesis in mood-related behavior initially stemmed from the findings that adult hippocampal neurogenesis is decreased by stress and increased by manipulations that counter the effects of stress, such as environmental enrichment and antidepressant treatment.

The first class of environmental factors found to impact adult neurogenesis was stress. Initially, it was found that stress affects proliferation during development. In rats, developmental neurogenesis can be downregulated by stress, as observed by decreased proliferation due to acute injection of corticosterone (CORT), a hormone released following stress (Gould et al. 1991), as well as due to exposure to predator odor (Tanapat et al. 1998).

This led to the hypothesis that *adult* hippocampal neurogenesis might also be downregulated by stress. This was first shown for acute administration of CORT in rats (Cameron and Gould 1994), and then later shown for acute psychosocial stress in tree shrews and monkeys (Gould et al. 1997, Gould et al. 1998). Other stressors have also been shown to decrease neurogenesis in the adult rodent hippocampus, including predator odor (Tanapat et al. 2001), daily restraint stress (Pham et al. 2003), and unpredictable chronic mild stress (Joels et al. 2004).

In addition to changes in the levels of adult hippocampal neurogenesis, stress has been shown to alter the proportion of cell types generated from neural stem cells in the adult SGZ. CORT, social isolation and aging have been shown to shift the balance towards fewer neurons, while exercise and enrichment shifts the balance towards more adult-born neurons produced (van Praag et al. 2005, Wong and Herbert 2006, Dranovsky et al. 2011, Chetty et al. 2014). To compensate for decreased neuronal production following stress, studies have observed increased numbers of stem cells (Dranovsky et al. 2011), astrocytes (van Praag et al. 2005), or oligodendrocytes (Chetty et al. 2014), but a full understanding of how stress affects the proportion of cells produced is lacking, along with the functional implications of these changes.

The mechanism through which stress impacts neurogenesis is not precisely understood, however there is evidence that it might be through regulation of the hypothalamic-pituitary-

adrenal (HPA) axis. The HPA axis controls the release of glucocorticoids, corticosterone (CORT) in rodents, from the adrenal glands, which act on many brain regions, along with other parts of the body. While CORT is released throughout the day, in a diurnal cycle of varying levels, stress elicits a large increase in CORT release, which changes its effects on the brain. The difference in the effects of baseline CORT versus stress-induced CORT is mediated through occupancy of two different receptors: mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs). MRs have a tenfold higher affinity for CORT, and are therefore well occupied by baseline CORT levels, while the higher levels of CORT that are released following stress mainly activate GRs (Reul and de Kloet 1985). GRs mediate the effects of stress on various brain regions, and also mediate negative feedback to the HPA axis (Herman et al., 1989). The hippocampus contains high levels of GRs, and is therefore particularly sensitive to changes in CORT levels, and involved in HPA axis regulation (Reul and de Kloet 1985).

Many stressors disrupt HPA axis regulation, including unpredictable chronic mild stress (Surget et al. 2011) and psychosocial stress (Fuchs and Flugge 1998). Additionally, a recent experiment has shown that when the effects of psychosocial stress on the HPA axis are prevented by removal of the adrenal glands (along with exogenous CORT provided at baseline levels), stress no longer decreases hippocampal neurogenesis (Lehmann et al. 2013). This study suggests that HPA axis activity mediates effects of stress on neurogenesis. Notably, neurogenesis is decreased by administration of high levels of exogenous glucocorticoids, either acutely (Gould et al. 1991, Cameron and Gould 1994) or chronically (Murray et al. 2008, David et al. 2009, Gourley and Taylor 2009). Together, these experiments suggest that increased release of glucocorticoids is both necessary and sufficient for the effects of stress on neurogenesis.

While stress decreases adult hippocampal neurogenesis, other conditions have been found to increase neurogenesis. The capacity for levels of adult hippocampal neurogenesis to be increased was initially discovered in rodents exposed to environmental enrichment (Kempermann et al. 1997) and exercise (van Praag et al. 1999).

Subsequently, antidepressants have been shown to increase proliferation of adult born neurons in the hippocampus of rodents (Malberg et al. 2000), monkeys (Perera et al. 2007), and humans (Boldrini et al. 2009). In rodents, antidepressants rescue the effects of stress on proliferation (Czeh et al. 2001), and speed up the maturation of adult-born neurons, as evidenced by increased dendritic arborization of immature, DCX-positive neurons, as well as increased ACSF-LTP (Wang et al. 2008). Alternative therapies for depression also increase neurogenesis in rodents, including electroconvulsive therapy (ECT) (Madsen et al. 2000) and transcranial magnetic stimulation (TMS) (Czeh et al. 2002).

Initial studies utilizing x-irradiation showed that adult hippocampal neurogenesis is required for the behavioral effects of antidepressants in both rodents (Santarelli et al. 2003) and non-human primates (Perera et al. 2011). Since these initial findings, subsequent studies in rodents have shown that adult hippocampal neurogenesis is necessary for some, but not all, of the behavioral effects of antidepressants (Surget et al. 2008, David et al. 2009). While these studies show a neurogenesis dependency of the behavioral effects of fluoxetine and imipramine, a selective serotonin reuptake inhibitor and tricyclic respectively, it should be noted that many other drugs have neurogenesis-independent antidepressant-like effects on behavior (David et al. 2007, Surget et al. 2008).

Similarly, some, but not all, of the beneficial behavioral effects of environmental enrichment depend on adult hippocampal neurogenesis. Environmental enrichment has been

shown to decrease anxiety-related behavior in a neurogenesis-independent manner (Meshi et al. 2006), but neurogenesis has been shown to be required for the ameliorative effects of environmental enrichment following social conflict stress (Schloesser et al. 2010).

In these initial studies, no effects were seen of ablation of neurogenesis on behavior at baseline, in the absence of antidepressants or environmental enrichment (Santarelli et al. 2003, Surget et al. 2008, David et al. 2009, Schloesser et al. 2010). However, since then, some studies have found that ablation of neurogenesis at baseline is sufficient to affect anxiety and depression-related behavior (Revest et al. 2009, Snyder et al. 2011), suggesting that in certain strains of mice tested under certain conditions, neurogenesis may affect baseline behavior.

It should be noted that there are inconsistencies between studies as to which behavioral tests are sensitive to levels of neurogenesis. For example, while a handful of studies have found a role for neurogenesis in the anxiety-based novelty suppressed feeding test (Santarelli et al. 2003, Surget et al. 2008, David et al. 2009, Snyder et al. 2011), other studies have reported effects in different anxiety-based tests, such as the elevated plus maze and light/dark test (Revest et al. 2009). These inconsistencies could be due to differences in strain, neurogenesis ablation method, or behavioral testing protocols, and despite these inconsistencies, the literature as a whole indicates a role for adult hippocampal neurogenesis in modulating anxiety and depression-related behavior.

It has been hypothesized that adult hippocampal neurogenesis is required for the behavioral effects of antidepressants through a role in HPA axis regulation. Regulation of the HPA axis has been a long-standing hypothesis of antidepressant action, supported by the repeated finding of correlations between improved patient prognosis and restored HPA axis regulation (Greden et al. 1983, Vreeburg et al. 2009). However, we note that no study to our

knowledge has shown whether changes in HPA regulation are necessary or sufficient for the effects of antidepressants on mood.

Due to the high levels of GRs present in the hippocampus (Reul and de Kloet 1985), this region is not only very sensitive to activity of the HPA axis, but also has been implicated as an important brain region that provides negative feedback to regulate the HPA axis. Selective knockout of hippocampal GRs results in a hyperactive HPA axis and depression-like behavior (Boyle et al. 2005). Furthermore, lesions of the hippocampus, hippocampal projection fiber tracts or specifically of the ventral subiculum, lead to impaired baseline or stress related HPA axis regulation (Herman et al. 1989, Herman et al. 1992, Herman et al. 1995). In this way, a loop is formed between the HPA axis, which releases CORT, and the hippocampus, which provides negative feedback that is necessary for normal HPA axis regulation.

A role for neurogenesis in regulating the HPA axis has been supported by findings that elimination of adult hippocampal neurogenesis increases the CORT response to acute stress (Schloesser et al. 2009, Snyder et al. 2011). Furthermore, another study found that chronic stress impairs HPA axis regulation, which is rescued by fluoxetine in a neurogenesis-dependent manner (Surget et al. 2011). The mechanism through which adult neurogenesis regulates the HPA axis is not known, but could occur through a cell-autonomous mechanism, as a subset of maturing adult-born cells have been shown to express GR, but not MR, perhaps allowing these cells to respond to CORT in a unique way (Cameron et al. 1993, Garcia et al. 2004), since the balance of MR and GR is thought to be important for maintaining neuronal homeostasis (De Kloet and Derijk 2004). Alternatively, non-cell autonomous effects on hippocampal activity levels may allow levels of adult hippocampal neurogenesis to regulate HPA axis activity independent of GR expression in adult-born cells, for example through changing dentate gyrus

activity levels. Interestingly, a study in which GR was knocked out of adult-born cells showed no effect on CORT levels at baseline or following contextual fear conditioning (Fitzsimons et al. 2013), suggesting a non-cell autonomous mechanism through which levels of adult hippocampal neurogenesis modulate the HPA axis.

Neurogenesis along the Dorsa/Ventral Axis

Increasing evidence suggests that along the longitudinal axis of the hippocampus, the effects of environmental conditions on levels of adult hippocampal neurogenesis varies, as does the functional role of adult-born neurons.

The effects of various environmental conditions on adult hippocampal neurogenesis have been shown to vary along the longitudinal axis. In rodents, some studies have found that stress decreases neurogenesis specifically or more severely in the ventral dentate gyrus (Tanti et al. 2012, Walker et al. 2014), while environmental enrichment has been shown to increase neurogenesis specifically in the dorsal dentate gyrus (Tanti et al. 2012). Additionally, agomelatine, a melatonin agonist and 5HT2C antagonist, has been found to specifically increase neurogenesis in the ventral hippocampus of rodents (Banasr et al. 2006), and has antidepressant-like effects on mood (Rainer et al. 2012). In humans, antidepressants appear to affect neurogenesis more robustly in the anterior hippocampus (which corresponds to the ventral hippocampus in rodents) (Boldrini et al. 2009).

Functionally, differential roles for adult hippocampal neurogenesis along the longitudinal axis has been supported by a recent study from the Hen lab in which x-irradiation was focused specifically to ablate adult hippocampal neurogenesis in either the dorsal or ventral half of the dentate gyrus. Mice with dorsal x-irradiation displayed impaired pattern separation, while ventral

x-irradiation prevented some of the behavioral effects of antidepressant treatment (Wu and Hen 2014). Together these studies suggest that neurogenesis in the dorsal and ventral regions of the hippocampus may be involved in memory and mood-related behavior respectively.

1.3 Hypotheses

As introduced in this chapter, ablation of adult hippocampal neurogenesis has shown that this process is required for certain memory related tasks, specifically involving pattern separation, as well as for some of the behavioral effects of antidepressants. Here, we hypothesize that increasing the levels of adult hippocampal neurogenesis in mice will impact both cognitive as well as anxiety and depression-related behavior, providing a target mechanism for future cognitive and mood enhancing treatments. Specifically the following hypotheses are tested:

1) Increasing adult hippocampal neurogenesis will improve performance on discrimination learning tasks, such as those in which performance is impaired in mice with ablated adult hippocampal neurogenesis.
2) Increasing adult hippocampal neurogenesis will be sufficient to affect anxiety and depression-related behavior.

Chapter 2: Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation performance at baseline, and increase exploratory behavior in mice exposed to voluntary exercise

2.1 Introduction

Adult hippocampal neurogenesis is a prominent feature of the mammalian hippocampus, and is responsive to environmental conditions such as age, stress, antidepressants and environmental enrichment (Gould et al. 1997, van Praag et al. 1999, Malberg et al. 2000, van Praag et al. 2005, Dranovsky et al. 2011). The necessity of adult hippocampal neurogenesis for various hippocampal-dependent functions in the cognitive and mood domains has been extensively tested by eliminating adult hippocampal neurogenesis using x-irradiation or genetic techniques. Together, these studies have shown that adult hippocampal neurogenesis is required for pattern separation (Clelland et al. 2009, Nakashiba et al. 2012, Tronel et al. 2012), proper HPA axis regulation (Schloesser et al. 2009, Snyder et al. 2011, Surget et al. 2011), and some of the beneficial effects of antidepressants and environmental enrichment on mood-related behavior (Santarelli et al. 2003, David et al. 2009, Schloesser et al. 2010).

Here, we sought to determine whether increasing adult hippocampal neurogenesis is sufficient to affect cognition and mood-related behavior. We generated a transgenic mouse model in which inducible deletion of the pro-apoptotic gene *Bax* specifically in neuronal stem cells and their progeny increases the number of adult-born neurons. We show that this genetic expansion is sufficient to enhance pattern separation in a fear discrimination learning paradigm; however, it is not sufficient to affect mood-related behavior in a battery of antidepressant-

sensitive behavioral tests. Furthermore, when combined with voluntary exercise, genetically increasing adult hippocampal neurogenesis is sufficient to increase exploratory behavior.

2.2 Methods

2.2.1 Mice

All mice used in experiments were homozygous for a loxP flanked *Bax* allele (Takeuchi et al. 2005). Mice that were also hemizygous for the Nestin-CreERT2 transgene (Dranovsky et al. 2011) are referred to as 'NCff' mice (for **N**estin-**C**reERT2;**f**loxed/**f**loxed), while mice without NestinCreERT2 are referred to as 'ff' mice. Since CreERT2 allows for *inducible* deletion of the *Bax* gene following treatment with tamoxifen, mice of this line are referred to as *iBax* mice (Figure 2.1a). The genotype of mice used for each experiment is labeled in the figures. This mouse line is maintained on a mixed C57BL/6 and 129/SvEv background.

Mice were 8-10 weeks old at the beginning of each experiment. Mice were housed 2-5 per cage and maintained on a 12 hour light/dark schedule with continuous access to food and water. All experiments were conducted with male mice, except for the voluntary exercise experiments (Figures 2.12-2.14). For the voluntary exercise experiment, female mice were group housed (4-5 mice) in a large cage (29.2 cm x 19.2 cm x 12.7 cm), each containing two running wheels, to which the mice had constant access. Female mice were used for this experiment because male mice have been observed to display increased aggressive behavior when housed in enriched cages, such as those used here (Marashi et al. 2003). All behavioral testing was conducted during the light cycle and with approval from the Institutional Animal Care and Use Committees at both Columbia University and the New York State Psychiatric Institute.

For **focal X-ray irradiation**, mice received three sessions, each separated by 3-4 days. 10 week old mice were anesthetized with sodium pentobarbital before each irradiation procedure. Mice were then placed in a stereotaxic frame and exposed to cranial irradiation in a Stabilopan X-ray system (Siemens), operated at 300 kVp and 20 mA. During the procedure, the animal's body was completely covered by a lead shield, except for a 3.22 mm x 11 mm window centered above the hippocampus (interaural 3.00 to 0.00). Dosimetry was conducted using an electrometer ionization chamber (model PF-06G, Capintec) and Ready Pack Radiographic XV films (Kodak). The corrected dose rate was approximately 1.8 Gy/min at a source-to-skin distance of 30 cm. The procedure lasted 2 min 47 seconds per animal per session, during which 5 Gy was delivered. Behavioral testing was conducted 4 months after x-irradiation.

2.2.2 Drug administration

Tamoxifen (TAM) was dissolved in a solution of corn oil (Sigma C8267) and 10% ethanol to a 10 mg/ml solution. 8-10 week old *iBax* mice received 2 mg TAM, or the same volume of corn oil and ethanol (vehicle), intraperitoneally once per day for 5 consecutive days.

Bromodeoxyuridine (BrdU) (150 mg/kg body weight, dissolved in .9% NaCl; Roche) was injected intraperitoneally (i.p.) to characterize levels of neurogenesis. Mice received BrdU injections once per day for 10 days to assess hippocampal neurogenesis (Figure 2.2), and once per day for 2 days to assess adult-born neurons in the olfactory bulb (Figure 2.3) at baseline. Mice exposed to voluntary exercise received BrdU injections once per day for 2 days (Figure 2.11).

2.2.3 Cognitive-related behavioral testing

One-trial contextual fear conditioning was conducted in a 20.3 cm x 15.9 cm x 21.3 cm chamber with two clear plexiglass walls, two aluminum walls and a stainless steel grid floor (one side of a shuttle box; Med-Associates, ENV-010MC), encased in a sound-attenuating box. On each day of testing, mice were allowed to habituate for 1 hour outside of the testing room before the experiment was started. During the test, behavior was recorded using digital video cameras mounted above the conditioning chamber, and analyzed using FreezeFrame and FreezeView software (Actimetrics). For the one-trial contextual fear conditioning protocol, 185 seconds after mice were placed in the chamber, they received a single 2 second food shock of .75 mA. The mouse was taken out of the chamber 15 seconds after termination of the foot shock, and returned to its home cage. For training context A, the fan and lights inside the chamber were on, the stainless steel grid floor was exposed, a mild lemon scent was used as an olfactory cue, 70% ethanol was used to clean the chamber between mice, and mice were brought into the testing room in a rectangular plastic cage. For the distinct context C, the stainless steel grid floor was covered with a plastic panel and cage bedding, the chamber walls were covered with plastic inserts, the house light and fan were turned off, the door to the sound-attenuating box was left ajar, letting in ambient light, a mild anise scent was used as an olfactory cue, and a non-alcoholic antiseptic was used to clean the chamber between mice. Mice were brought into the testing room in pie shaped cages by a different handler than for the training context A, and the testing room was dimly lit during placement of mice in the testing chamber.

Contextual fear discrimination learning. In this test, mice were exposed to the shock context A and a similar context B daily, as diagrammed in figures 2.5d, 2.7a and 2.8a. The shock-associated training context A and the similar no-shock context B shared many features,

including the exposed stainless steel grid floor. Context A was the same as described above for one-trial contextual fear conditioning. Context B differed from Context A in that two plastic inserts were used to cover the walls, the light and fan inside the chamber were turned off, the chamber door was left ajar during testing, a mild mint scent was used as an olfactory cue, a non-alcoholic antiseptic was used to clean the box between mice, and mice were brought into the testing room in buckets. The shock protocol used here in context A was the same as for one-trial contextual fear conditioning. In the similar context B, mice were left in the box for 180 seconds with no shock. Freezing levels in both context A (3 minutes preshock) and context B (3 minutes) were recorded and analyzed each day.

Extinction learning and reinstatement: Mice were subjected to a single 2 second foot shock (.75 mA) after 185 seconds in context A (as described above). For the following six consecutive days (Days 1-6, Figure 2.9), mice were placed back into context A once daily, for 3 minute re-exposure sessions without foot shocks. On day 7, mice received a single foot shock in a novel context C (as described above) and reinstatement of freezing behavior in context A was assessed 24 hours later.

2.2.4 Anxiety and depression-related behavioral testing

Psychiatric disorders under the anxiety and depression umbrellas are thought to represent heterogeneous patient populations with various genetic and environmental factors contributing to the disease; however, it is the symptomatic behavior of these individuals that defines their diagnosis. Therefore, when modeling and testing for these conditions, we are particularly interested in behavior relating to anxiety and depression.

While anxiety and depression-related behavioral categories can be dissociated, many manipulations, such as chronic antidepressant treatment, affect behavior in both categories of tests (Dulawa et al. 2004). Similarly, in patients, there is high comorbidity between depression and anxiety disorders, as studies have found that around 40-50% of patients with depression also have diagnosed anxiety disorders (Sanderson 1990, Fava 2000). This high level of comorbidity may be a result of overlapping neural circuitry underlying these disorders, and it is perhaps not surprising that many manipulations in rodents affect behavior in both of these domains.

Anxiety disorders are generally characterized by excessive fear and avoidance, either broadly (such as in generalized anxiety disorder), or to selective cues (such as in specific phobias). These avoidance symptoms have been modeled in rodents using conflict-based tasks, in which subjects must choose between relatively safe and unsafe areas of an arena to which they have free access (Bailey and Crawley 2009). In these tasks, there is a conflict between a rodent's tendency for exploratory investigation versus aversive components present in parts of the arena (such as bright light). In general, these tests have face validity based on the proposition that many physiological and behavioral responses to fear are evolutionarily conserved between rodents and humans, such as release of glucocorticoids and avoidance of fearful places (Cryan and Holmes 2005). These tests also have predictive validity based on the effects of anxiolytics, such as benzodiazepines. A few commonly used conflict-based anxiety tasks, from which data will be presented throughout the thesis, are discussed.

In the **open field test**, a mouse is placed in a novel, open arena. In this task, the amount of time and distance traveled is calculated in a defined center region, as well as for the rest of the arena. On the one hand, rodents have been found to generally prefer closed and dark spaces over open and brightly lit spaces, which would predict that mice would spend all of their time along

the edges of the arena, away from the center region. On the other hand, a propensity for mice to explore novel spaces has been observed, which would predict that they would spend time exploring the center region. These two drives produce a conflict for the rodent, which must spend its time in either the center or peripheral regions. Chronic stress decreases time and distance in the center region, while anxiolytics have the opposite effect (although notably, these effects are not always seen) (Prut and Belzung 2003), providing both face and predictive validity.

Here, the open field test was conducted in a square 43.2 cm x 43.2 cm enclosure with infrared beams to detect animal movement (MED Associates), for either 30 or 60 minutes. The center region was defined by the inner 21.2 cm x 21.2 cm area. Distance, time in center, center entries and rearing were analyzed by MED Associates software. Percent center distance was calculated as the percent of total distance travelled in the center region.

The **light/dark test** is a variant of the open field test, in which the arena is separated into two compartments: one which is dark and covered, and the other, which is light and open. In this test, mice are initially placed in the dark compartment. Similar to the open field, the mouse is thought to have conflicting drives, to remain in the innately preferred dark compartment or to explore the novel, light compartment. The latency to transition into the light compartment, the number of transitions between compartments, and the amount of time the rodent spends in the light compartment, are assayed as measures of anxiety-related behavior, which are affected by models of stress (Ardayfio and Kim 2006), and anxiolytics (Crawley 1981, Belzung et al. 1987, Bourin and Hascoet 2003).

Here, the light/dark test was conducted in the same chambers as for the open field. A dark plastic box with opaque sides and ceiling was placed over half of the open field arena, with an opening at floor level, allowing the mouse to pass between dark and light compartments. Mice

were initially placed in the dark compartment, and allowed to freely move throughout the arena for five minutes. Movement was detected by infrared beams, which could pass through the opaque plastic box, to measure ambulatory distance, time, and entries for each compartment.

In the **elevated plus maze**, mice are placed in a plus shape arena with four arms, elevated above the floor. Two opposing arms have tall walls (referred to as the 'closed arms'), while the other two arms have no walls (referred to as the 'open arms'). This test comprises a similar conflict to the open field and light/dark tests, where the closed arms are analogous to the edges of the open field or the dark compartment of the light/dark test, while the open arms are analogous to the anxiogenic center region or light compartment. Mice will typically spend the majority of their time in the closed arms, but will explore the open arms to varying extents. Much previous work has shown that behavior in this test is sensitive to anxiolytic and anxiogenic substances, which respectively increase and decrease time spent in the open arms (Pellow et al. 1985, Pellow and File 1986, Lister 1987, Walf and Frye 2007).

Mice were tested in the elevated plus maze for five minutes. The maze used has arms 7.6 cm wide, 28 cm long and 31 cm above the floor. Two opposing closed arms have 15 cm high walls, while two opposing open arms have a 1 cm high lip. Testing was video recorded and later analyzed by an observer (blind to genotype) for time spent and entries into the open arms.

In the **novelty suppressed feeding test**, mice are placed into a novel, brightly lit, rectangular arena containing a food pellet on a raised platform in the center of the arena. This test is conducted after food deprivation, and is a conflict-anxiety test where animals must enter the anxiety-provoking center of the arena for food. The latency to take a bite of the food pellet is assessed as the main measure of anxiety. Latency is increased in models of stress, such as chronic CORT treatment (David et al. 2009), and interestingly, this test is specifically sensitive

to chronic, but not acute, treatment with antidepressants, which decrease latency (Santarelli et al. 2003).

Here, mice were food deprived in their home cages for 24–26 h before testing. The testing apparatus consisted of a plastic arena (45 cm long, 15 cm high and 30 cm wide) with the floor covered by about 2 cm of wood-chip bedding. A single food pellet (familiar laboratory mouse chow) was placed on a circular piece of white filter paper (12 cm in diameter) in the center of the arena. Mice were placed in a corner of the arena, and the latency to begin feeding on the food pellet was recorded (up to a maximum time of 10 minutes). Testing was conducted under bright light conditions. Each mouse was weighed before food deprivation and just before testing to assess changes in body weight during food deprivation. Immediately after the test, each mouse was transferred to its home cage, and the amount of food consumed within 5 minutes was assessed.

Tests of depression-related behavior generally have been characterized either by predictive validity in that behavior of rodents is affected following treatment with antidepressants, or by face validity in that the tests appear to assess a phenotype observed in human patients, including behavioral despair and anhedonia.

The **forced swim test** was one of the earliest rodent behavioral tests found to be sensitive to antidepressants (Porsolt et al. 1978, Petit-Demouliere et al. 2005). In this test, mice are placed in a beaker of room temperature water for two sessions on subsequent days. Animals will initially swim for the majority of the first session. However, in the second session, animals typically display floating behavior. The amount of time mice spend floating in the second session (referred to as immobility time) has been interpreted as a measurement of learned helplessness, or the willingness of an animal to give up. Although this test has sometimes been considered as

only a screen for antidepressants (Gardier and Bourin 2001), it is also sensitive to various procedures thought to induce depression-like behavior, such as chronic mild stress (Molina et al. 1994, Bielajew et al. 2003), thereby validating this test as relevant to depression-related behavior (Cryan et al. 2005).

For the forced swim test, mice were placed in clear plastic buckets (19 cm diameter and 23 cm deep) filled with water (23–25 °C) for 6 minutes, and their behavior was recorded using an automated video-tracking system. Testing was carried out over two consecutive days, with the first day serving the purpose of pre-exposure. Mobility (swimming and climbing behavior) was analyzed using ViewPoint Life Sciences Software.

The **tail suspension test** is also widely used to assess antidepressant-like activity in mice (Cryan et al. 2005). In this test, mice are suspended by their tails for five minutes. As animals are placed in this inescapable position, they initially display attempted escape behavior and then transition to an immobile, hanging posture, often referred to as a state of behavioral despair. Like in the forced swim test, mobility is interpreted as a measurement of this escape-related behavior and ability to cope in a stressful situation. This test has been validated primarily by the finding that many antidepressants increase mobility time (Steru et al. 1985). Furthermore, mice with high levels of immobility in this test have been used to breed 'helpless' mice, which are sensitive to antidepressants and display serotonergic dysfunction similar to that found in human patients with depression (Vaugeois et al. 1996, El Yacoubi et al. 2003). Here, mice were suspended from a table-top by their tails for 5 minutes. This test was video recorded and mobility was analyzed by ViewPoint Life Sciences software.

2.2.5 Plasma corticosterone

HPA axis activity was assessed in two paradigms. In the first, mice were taken from their home cage, placed in a decapicone plastic restraining device (Braintree Scientific) for 30 minutes, and then placed in a novel cage (as in (Snyder et al. 2011)). Blood was taken either immediately after removal from the home cage (before restraint), immediately after restraint, or after 30 or 60 minutes in the novel cage (following restraint). Different groups of mice were used for each time point. Blood was collected via submandibular bleed using a Goldenrod Animal Lancet (MEDIpoint), into a tube containing EDTA. Blood was centrifuged at 2000 rpm for 5 minutes for separation of plasma, which was stored at -20°C until assayed using a commercially available EIA kit (Arbor Assays).

In the second paradigm, mice were placed in a novel cage without bedding for 15 minutes (as in (Schloesser et al. 2009)). Blood was collected immediately afterwards, and treated as described above.

2.2.6 Electrophysiological recordings

For electrophysiological recordings, brains were collected from animals after inducing deep anesthesia with halothane, followed by decapitation. Transverse hippocampal slices (400 µm) were cut on a vibratome. Slices were incubated in an interface chamber at 32 °C and perfused with oxygenated artificial cerebrospinal fluid (ACSF) (containing 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 26.2 mM $NaHCO_3$, 1 mM NaH_2PO_4 and 11 mM glucose). Slices equilibrated for 2 hours before positioning electrodes and beginning stimulation. To record from the dentate gyrus, the medial perforant path (MPP) was stimulated using a stimulation isolation unit and a bipolar tungsten electrode (World Precision Instruments). Evoked

potentials were recorded in the molecular layer above the upper blade of the dentate gyrus using a glass capillary microelectrode filled with ACSF (and with a tip resistance of 1–3 MΩ). Isolation of the MPP was confirmed by assessing paired-pulse depression of the MPP–dentate gyrus synaptic connection at 50 ms, which generated the highest level of depression. Input–output curves were obtained after recordings had been stable for 10 min. The stimulation intensity that produced one-third of the maximal response was used for the test pulses and tetanus. After a stable baseline response to test stimulation (once every 20 s) had been observed for 15 min, the ability to elicit LTP was assessed. LTP was induced with a weak stimulation protocol consisting of four trains of 1 s each, at 100 Hz within the train, repeated every 15 s. Responses were recorded every 20 s for 60 min after LTP induction. A similar protocol was used to elicit and record LTP of mature dentate granule neurons except that 10 μM bicuculline (bicuculline methobromide, Sigma, B7561) was added to the ACSF to block GABAA receptors.

2.2.7 Immunohistochemistry

At sacrifice, mice were anesthetized with ketamine and xylazine (100 and 7 mg/kg respectively). Mice were then transcardially perfused with cold saline and 4% paraformaldehyde. Brains were postfixed in 4% paraformaldehyde overnight, and then transferred to 30% sucrose for cryoprotection. Brains were coronally sectioned (35 um) throughout the hippocampus.

For doublecortin (DCX) and Ki67 immunohistochemistry (stained separately), tissue was washed in PBS, quenched for endogenous peroxidases with 1% hydrogen peroxide in PBS/methanol, washed in PBST (PBS with .3% Triton), blocked for 1 hour at room temperature in PBST and 10% normal donkey serum, and then incubated in primary antibody (goat anti-DCX [Santa Cruz, SC-8066] 1:500 or rabbit anti-Ki67 [Vector Laboratories, VP-RM04] 1:100 in

PBST) overnight at 4°C. Sections were then washed in PBS and incubated in secondary antibody (biotin donkey anti-goat or anti-rabbit 1:500 [Jackson ImmunoResearch] in PBS) for 2 hours at room temperature. Sections were washed in PBS, incubated for 1 hour with avidin/biotin (ABC kit, Vector Laboratories), washed in PBS, and then incubated with DAB peroxidase (Vector Laboratories) for 5 minutes. Sections stained for Ki67 were incubated in Nuclear Fast Red (Vector Laboratories) for 5 minutes after they were mounted onto slides. An investigator blind to treatment counted every 6th section throughout the dentate gyrus for both the total number of DCX-positive neurons, and the number of DCX-positive neurons with tertiary dendrites. Similarly, the total number of Ki67-positive cells was counted in the granule cell layer and subgranular zone of the dentate gyrus of every 6th section. Here, Fast Red was used to determine the boundaries of the granule cell layer. Analysis was conducted and images were taken using a Zeiss Axio Observer A.1 microscope.

For BrdU and NeuN immunohistochemistry, sections were mounted onto SuperFrost Plus charged glass slides, and then heated for 1 hour in citric acid buffer for antigen retrieval, washed in PBS, blocked in PBST and 10% normal donkey serum for 2 hours at room temperature, and then incubated in primary antibody (rat anti-BrdU [Serotec OBT0030] 1:100 and mouse anti-NeuN [Chemicon MAB377] 1:500 in PBST) overnight at 4°C. The following day, sections were washed in PBS and then incubated in secondary antibody (donkey anti-rat Cy3 and donkey anti-Mouse Cy5 [Jackson ImmunoResearch] 1:200 in PBS). An investigator blind to treatment counted every 6th section throughout the dentate gyrus for the total number of BrdU-positive cells using a Zeiss Axiovert 200 microscope. For quantification of survival of adult-born cells in the main olfactory bulb, two 20x magnification images of randomly selected regions in the granule cell layer were taken from six matched sagittal sections for each mouse. BrdU-positive

cells were quantified using a cell counter plug-in for the software ImageJ (NIH), and surface density was computed. BrdU and NeuN colabel analysis was conducted by scanning z-stacks of BrdU-positive cells from the hippocampus of each mouse on an Olympus Fluoview 1000 confocal microscope at 60x magnification.

2.2.8 Statistical methods

Statistical analysis was conducted using StatView software or Microsoft Excel. Statistical significance was assessed by unpaired two-tailed Students *t* test or ANOVA and Fisher's predicted least-square difference test for *post hoc* analysis. Significance was set at $p<0.05$.

2.3 Results

2.3.1 Characterization of *iBax* mice

To genetically increase levels of adult neurogenesis, we utilized a bi-transgenic mouse model that expresses the inducible recombinase CreERT2 under the Nestin promoter (Dranovsky et al. 2011), as well as floxed alleles of the pro-apoptotic gene *Bax* (Takeuchi et al. 2005). In this mouse line, tamoxifen (TAM) treatment induces deletion of three exons in the *Bax* gene, specifically in nestin-expressing neural stem cells, and therefore also in their progeny (*iBax* mice). In these cells, no functional BAX protein is produced. Normally, 60-80% of adult-born dentate granule neurons undergo cell death before reaching maturity through a process that requires BAX (Dayer et al. 2003), therefore we hypothesized that deletion of *Bax* in these cells would increase levels of adult hippocampal neurogenesis via increased survival (Fig 2.1). We note that although some ectopic expression has been reported in the Nestin:CreERT2 transgenic

mouse line (Sun et al. 2014), it is unlikely to influence our phenotype since these ectopic cells are not undergoing apoptosis, and are therefore unlikely to be affected by *Bax* deletion.

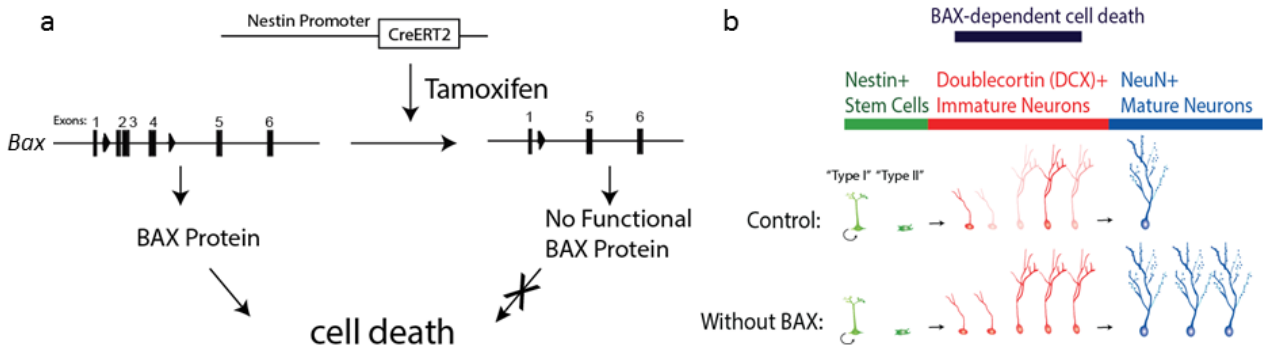

Figure 2.1 Adult hippocampal neurogenesis in *iBax* mice.
a) Genetic strategy to inhibit cell death. The floxed *Bax* allele contains *loxP* sites before exon 2 and after exon 4. In the absence of tamoxifen, this *Bax* allele produces normal BAX protein, which promotes cell death. In mice that express CreERT2 under the Nestin promoter, tamoxifen enables CreERT2 to excise exons 2-4 of the floxed *Bax* allele, no functional BAX protein is produced, and therefore cell death is inhibited. Schematic of the *Bax* gene was adapted from (Takeuchi et al. 2005). b) Schematic illustrating increased adult hippocampal neurogenesis in mice lacking BAX. Nestin-expressing Type I and Type II stem cells (green) divide to produce adult-born granule cells. In the adult dentate gyrus of control mice, a substantial fraction of adult-born neurons undergo BAX-dependent cell death during maturation (pale red). Nestin-CreERT2 mediated ablation of *Bax* results in the generation of adult-born neurons that lack BAX, thereby preventing their death and increasing the number of immature (red) and mature (blue) adult-born neurons.

Analysis of adult hippocampal neurogenesis was conducted by immunostaining for the immature neuronal marker doublecortin (DCX) in mice carrying one Nestin-CreERT2 and two floxed Bax alleles ("NCff" mice) that were injected with Vehicle or TAM. In mice sacrificed four weeks after vehicle or TAM injection, we see that TAM treated mice have a small increase in the number of DCX-positive neurons with tertiary dendrites, which represents a mature subset of DCX-positive neurons ($p=0.05$) (Figure 2.2 a,b). By eight weeks after vehicle or TAM injection, we observed that TAM treated animals have a significant increase in both the total

number of DCX-positive neurons (1.8 fold increase, $p = 0.038$), as well as the total number of DCX-positive neurons with tertiary dendrites (2 fold increase, $p = 0.006$) (Figure 2.2 c,d). The larger increase in the number of DCX-positive cells at 8 weeks compared to 4 weeks following TAM injection is consistent with the fact that CreERT2 mediated recombination occurs in both the slowly dividing self-renewing Type I neural stem cells as well as in Type II transient amplifying cells. The expansion of adult hippocampal neurogenesis in *iBax* mice is comparable to, if not greater than, that observed following chronic antidepressant treatment (Malberg et al. 2000, Santarelli et al. 2003, Li et al. 2008, David et al. 2009), environmental enrichment (van Praag et al. 2000), and exercise (van Praag et al. 2000).

Since BAX is involved in cell death, we expected that levels of neurogenesis would be increased by changes in survival rather than proliferation. The rate of proliferation was assessed with immunostaining for Ki67, a cellular marker of proliferation. As expected, we saw no effect of TAM treatment on Ki67 levels 4 or 8 weeks following TAM treatment (Figure 2.2 e-f). The rate of survival was assessed using BrdU pulse-chase labeling. Here, BrdU was injected over ten consecutive days, and mice were sacrificed six weeks later. We saw a 3.6 fold increase in levels of BrdU-labeled cells in mice treated with TAM compared to controls ($p = 0.0004$) (Figure 2.2 g,h). We assessed the percent of BrdU-positive cells that are colabeled with the mature neuronal marker NeuN, and found similar levels in both groups of mice (Figure 2.2 h).

BrdU pulse-chase labeling was also used to assess neurogenesis in the olfactory bulb. Here, BrdU was injected over two consecutive days, and mice were sacrificed eleven weeks later. We saw a 1.6 fold increase in the levels of BrdU-labeled cells in mice treated with TAM compared to controls ($p = 0.01$) (Figure 2.3). This represents a significant increase, but less than that observed in the hippocampus.

Further characterization of this line has shown no difference between groups in dentate granule cell markers, granule cell layer volume, apical dendrite maturation or axonal extension and targeting in CA3 (Sahay et al. 2011), suggesting that aside from the increase in adult-born cells, the dentate gyrus maintains normal structure.

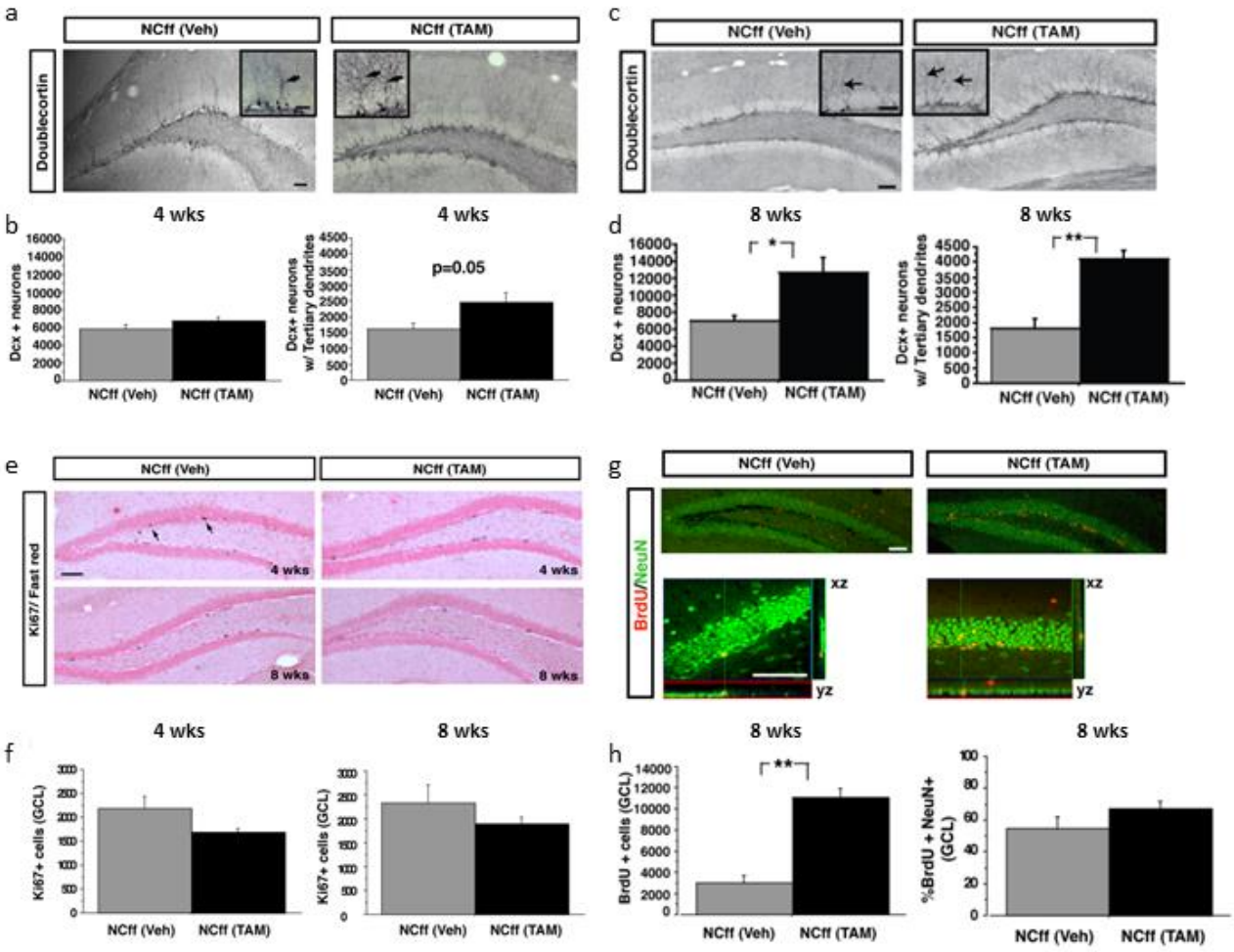

Figure 2.2 TAM increases hippocampal neurogenesis in *iBax* mice.
a,c) Representative images of DCX immunostaining in mice sacrificed 4 (a) or 8 (c) weeks after vehicle or TAM administration. Higher magnification insets include arrows indicating DCX-positive neurons with tertiary dendrites. Scale bars 100 µm. b) Quantification of DCX at 4 weeks reveals no significant difference in the total number of DCX-positive neurons (5,866±4,344 (Vehicle), 6,787±409 (TAM), (p>0.05)), but a trend for increased DCX-positive neurons with tertiary dendrites in TAM treated animals (1,620±187 (Vehicle), 2,469±305 (TAM), (p=0.05)). n=4 mice/group. d) Quantification of DCX at 8 weeks reveals that TAM treatment significantly increases both the total number of DCX-positive neurons as well as the number of DCX-positive neurons with tertiary dendrites. (Total DCX-positive neurons: 6,974±600 (Vehicle), 12,636±1,764 (TAM) (p =0.038). DCX-positive neurons with tertiary dendrites: 1,800±340 (Vehicle), 4,090±285 (TAM) (p=0.006)). n=3 mice/group. e) Representative images of Ki67 immunostained coronal hippocampal sections in mice sacrificed 4 or 8 weeks after vehicle or TAM administration. Scale bar = 100 µm. f) There are no significant differences in Ki67 between groups at either 4 weeks (p=0.1, n=4 mice/group), or 8 weeks (p=0.3, n=5 mice/group) post vehicle or TAM administration. g) Representative images of BrdU and NeuN immunostaining. Scale bars 100 µm (top), 50 µm (bottom). h) TAM treatment increases the number of BrdU-positive cells in the granule cell layer (GCL) (3,004±733 (Vehicle), 11,113±874 (TAM), (p=0.0004)). There is no difference between groups in the percent of BrdU-positive cells that are also NeuN-positive (54.6±7.3% (Vehicle), 67.2±4.7% (TAM)). *p<0.05, **p<0.01. Results are presented as mean ± SEM.

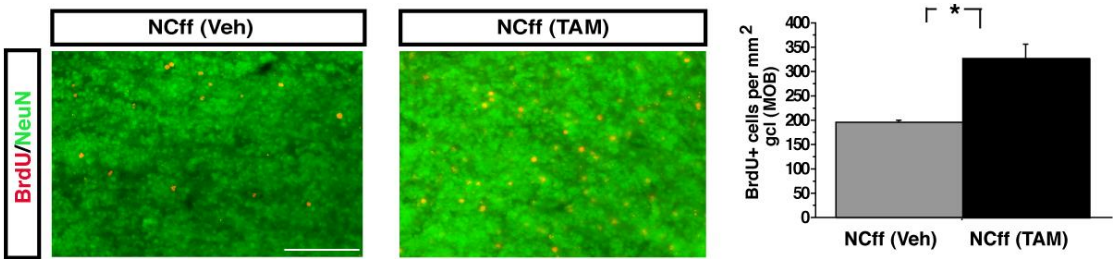

Figure 2.3 TAM increases neurogenesis in the olfactory bulb in *iBax* mice.
Representative images of BrdU and NeuN-labeled cells in the olfactory bulb (left). Scale bar = 100 µm. TAM treatment significantly increases the density of BrdU-positive cells (196.14±4.5 (Vehicle), 328±29 (TAM), p=0.01), n=3 mice/group. *p<0.05. Results are presented as mean ± SEM.

To assess the functional contribution of increased levels of adult hippocampal neurogenesis, we performed electrophysiological experiments, assessing long-term potentiation (LTP) at medial entorhinal perforant path to granule cell synapses. This synapse has been previously shown to exhibit strong LTP in the presence of bicuculline. In the absence of bicuculline, a much weaker LTP is observed (referred to as ACSF-LTP), which is abolished in the absence of adult hippocampal neurogenesis (Snyder et al. 2001, Saxe et al. 2006). Here, we observed that slices from TAM treated animals display increased ACSF-LTP ($p = 0.039$) (Figure 2.4, top panel), while there is no difference between groups for LTP in the presence of bicuculline, a GABA A receptor antagonist (Figure 2.4, bottom panel), a form of LTP mediated by mature granule cells. These findings suggest that increasing the number of adult-born neurons is sufficient to enhance neurogenesis-dependent LTP, and that additional adult-born neurons in TAM-treated *iBax* mice functionally integrate into the hippocampal network.

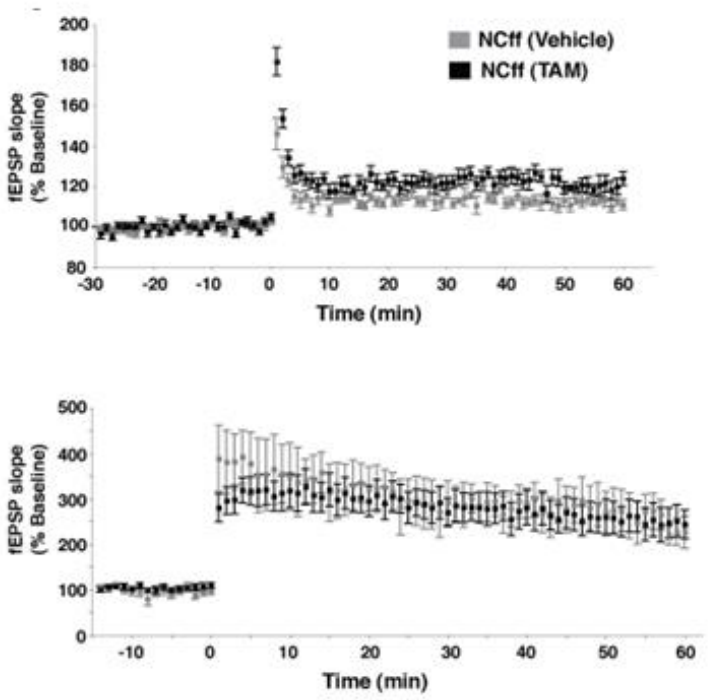

Figure 2.4 TAM treated *iBax* mice show enhanced ACSF-LTP.
TAM treatment increases ACSF-LTP over vehicle treated animals (top panel), (p=0.039), n=8-11 slices from 6-7 mice/group. There is no difference in LTP in the presence of bicuculline (bottom panel), n=4-6 slices from 3 mice/group. Results are presented as mean ± SEM.

2.3.2 Levels of adult hippocampal neurogenesis impact performance in a fear-based pattern separation task.

Since the dentate gyrus has been shown to play a role in pattern separation (Marr 1971, O'Reilly and McClelland 1994, McHugh et al. 2007), we were interested in exploring the role of adult hippocampal neurogenesis in this process. To do this, we used fear discrimination learning as a behavioral test thought to require pattern separation (McHugh et al. 2007), and tested the effects of ablation of neurogenesis in mice that underwent x-irradiation, as well as the effects of increased neurogenesis in *iBax* mice. In order to rule out effects on encoding or retrieval, we also tested both groups of mice in a single-trial contextual fear conditioning paradigm.

Many rodent learning and memory tasks involve associative learning processes that elicit quantifiable behavioral responses. One commonly used paradigm is fear conditioning, through which a rodent learns to associate an aversive unconditioned stimulus (for example a foot shock) with a conditioned stimulus (such as a context or a tone) which is initially of neutral salience.

Contextual fear conditioning has been shown to be hippocampal-dependent (Phillips and LeDoux 1992). In this test, animals are trained by receiving shocks in a specific context. When animals are re-exposed to the context in which they were shocked, they normally display freezing behavior, which increases with shock intensity (Curzon et al. 2009). The percent of time that an animal freezes during re-exposure to a shock-paired context is therefore interpreted as the strength of the memory of the shock experience. Importantly, following contextual fear conditioning, animals do not display freezing behavior when exposed to a novel context, suggesting that freezing behavior is specifically associated with the shock context.

Mice that underwent hippocampal irradiation have very low levels of neurogenesis. X-ray significantly decreases the number of DCX-positive neurons as compared to sham treated animals ($p=0.011$) (Figure 2.5 a,b). Irradiated mice and sham controls were first tested in a single trial fear conditioning paradigm. When mice were shocked in context A and tested in that same context 24 hours later, both x-ray and sham groups displayed similarly increased freezing levels (Figure 2.5 c), suggesting that both groups encode and express fear of context A equally.

Sham and irradiated mice were then tested in contextual discrimination learning, where each day they were exposed to both the shock context A, as well as a similar, but safe, no shock context B (Figure 2.5 d). The contextual fear discrimination learning task has been developed as a variant of contextual fear conditioning, to test an animal's ability to distinguish between similar experiences (Sahay et al. 2011). In this task, animals are exposed to two similar contexts daily,

one in which they receive a shock, the other in which they do not. Freezing behavior is measured during exposure to both contexts, and initially, animals generally display similarly high freezing levels in both contexts. However, after several days, animals tend to display higher freezing levels in the shock context than in the similar, no-shock context. The difference in freezing levels between the two contexts has been interpreted as an ability of the animal to distinguish between the two similar contexts, which is thought to require pattern separation.

In the contextual discrimination learning task, freezing in each context was used to represent the relative fear expressed by mice, from which an assessment of whether they have learned to distinguish between the two contexts can be extrapolated. While initially, both sham and irradiated groups of mice displayed equal freezing levels in both contexts A and B (Figure 2.5 e,f), by day 5, sham mice displayed a more robust difference in freezing between the two contexts compared to irradiated mice ($p=0.04$) (Figure 2.5 f).

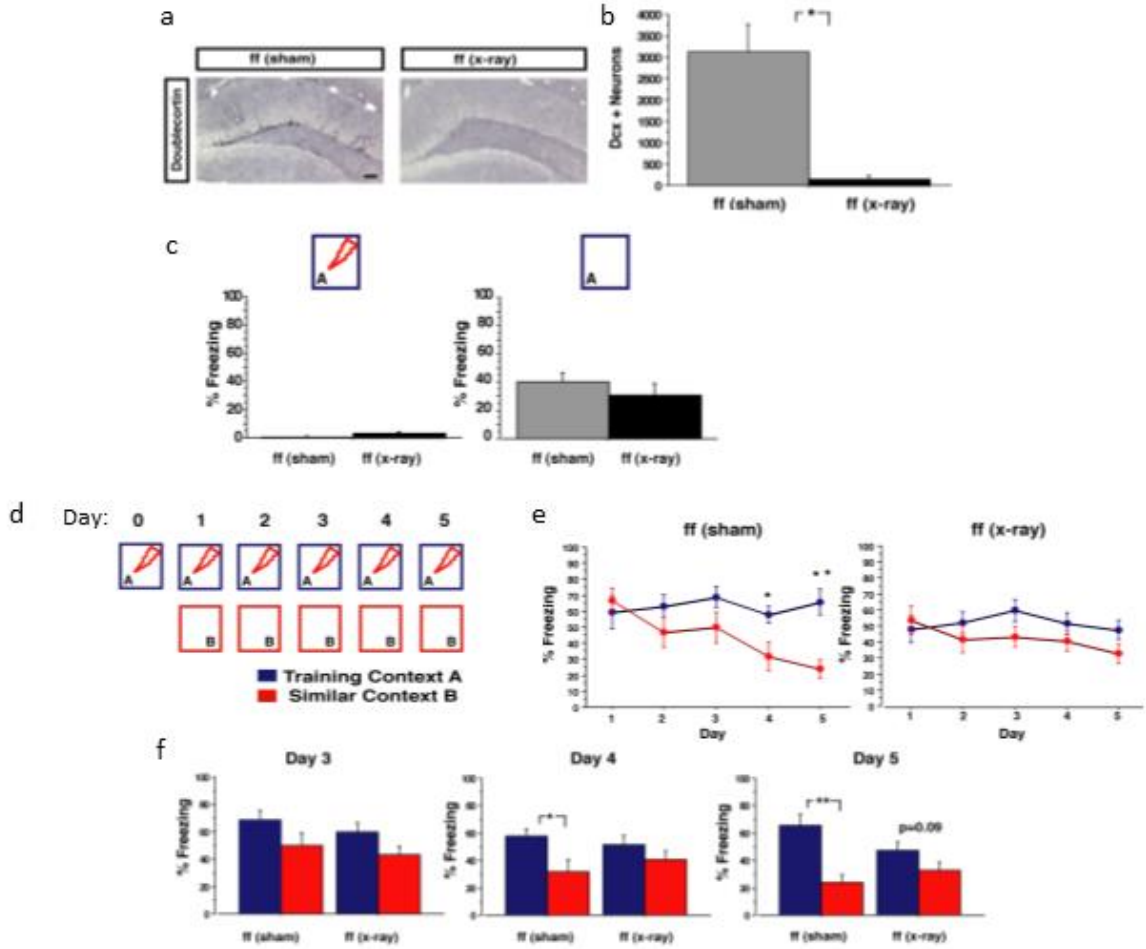

Figure 2.5 Ablation of adult hippocampal neurogenesis impairs performance in a fear discrimination learning task.

a) Representative images of DCX immunostaining in coronal hippocampal sections. Scale bar 100 µm. b) Quantification of DCX shows that x-irradiation greatly diminishes neurogenesis. (Total DCX-positive neurons: 3,120±659 (sham), 144±75 (x-ray) (p=0.011)), n=3 mice/group. c) There is no difference in freezing levels between x-ray and sham treated mice before or after contextual fear conditioning using a single foot shock-context pairing protocol (p>0.05), n=8-9 mice/group. d) Contextual fear discrimination learning paradigm. e) Freezing levels over days in each context, separated by treatment group, reveal a significant difference in freezing between contexts over days in the sham, but not x-ray group. (Sham p=0.004. X-ray p=0.1) f) Freezing levels of each group in each context on days 3, 4, and 5. By day 5, sham mice display a more robust discrimination between the two contexts as compared to x-ray mice (p=0.04). *p<0.05, **p<0.01. Results are presented as mean ± SEM.

Next we used *iBax* mice to assess the effect of increasing adult hippocampal neurogenesis on contextual fear conditioning and discrimination learning. 24 hours after fear conditioning in context A, both TAM and vehicle treated mice displayed similar high freezing

levels (Figure 2.6 b), suggesting that both groups acquired and retained contextual fear conditioning equally well. Furthermore, both groups of mice froze at low levels when tested in a distinct context C that is very different from the training shock context A (Figure 2.6 b), suggesting that increasing adult hippocampal neurogenesis does not impact an animal's ability to distinguish between two distinct contexts.

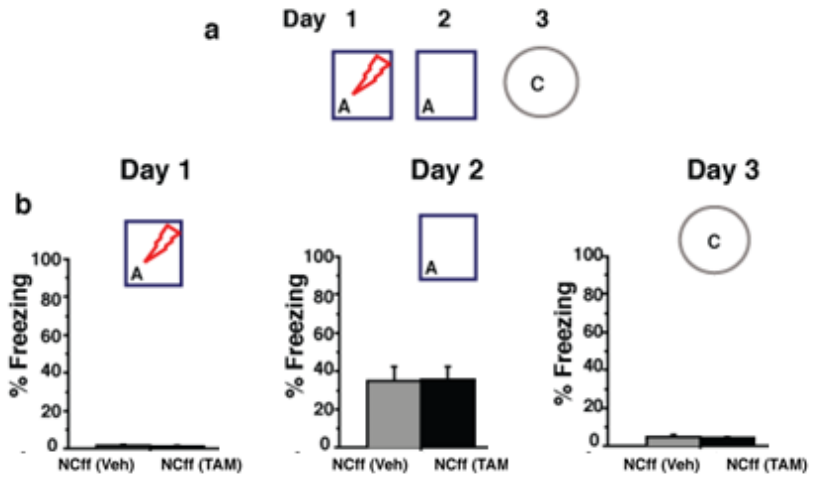

Figure 2.6 Increasing adult hippocampal neurogenesis does not affect one-trial contextual fear conditioning. a) Experimental design. b) Mice initially display low freezing levels in context A on Day 1, before training. On Day 2, high levels of freezing were observed in both groups, with no significant difference between groups ($p>0.05$). On Day 3, mice show low levels of freezing in the distinct context C. Both groups show significantly lower freezing in context C than context A ($p<0.0001$). Results are presented as mean ± SEM.

Next, we tested pattern separation in mice with increased neurogenesis using a contextual fear discrimination learning task. As before, this paradigm initially consisted of daily presentations of the shock context A, following by presentation of the similar context B. Additionally, to make the task more difficult, the order of context presentations was reversed on day 7. Both groups displayed significantly different freezing levels between the two contexts over days (Vehicle: $p<0.0001$; TAM: $p=0.0002$) (Figure 2.7 b). *Post hoc* tests revealed more

days where freezing between contexts was significantly different in TAM compared to vehicle treated mice, especially after the order of context presentation was reversed (days 7-9, Figure 2.7 b).

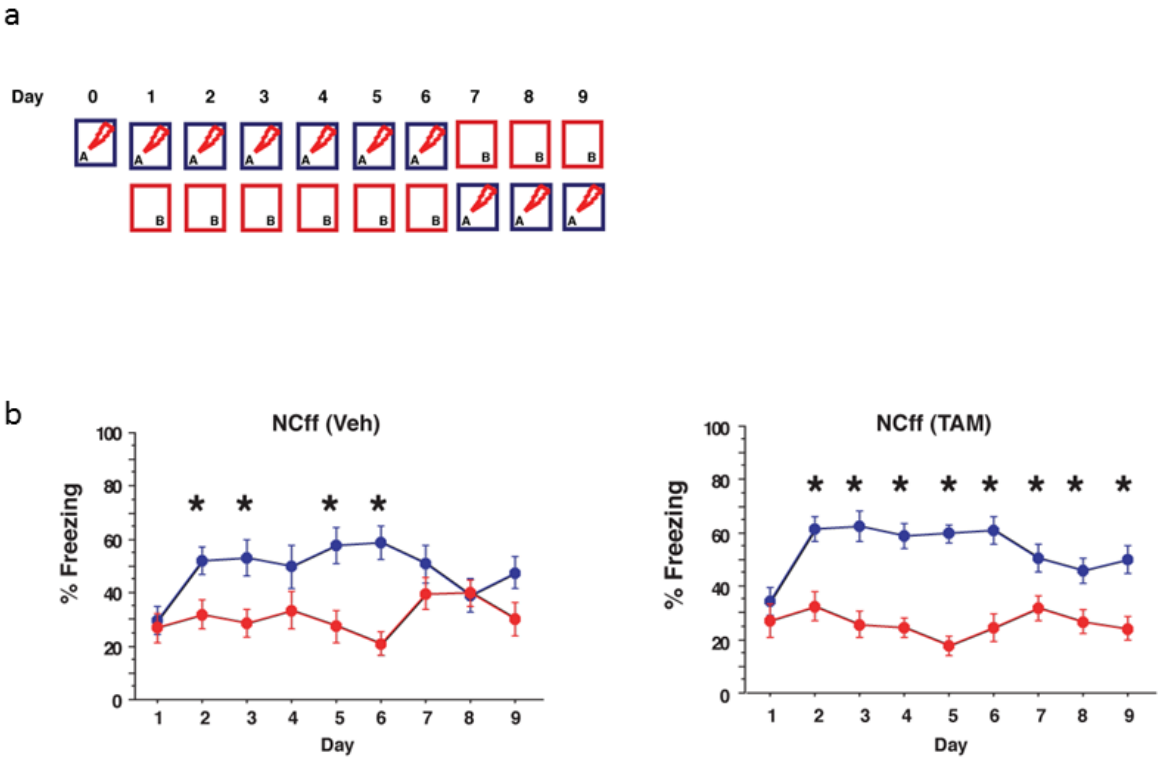

Figure 2.7 Increasing adult hippocampal neurogenesis improves performance in a fear discrimination learning task.
a) Contextual fear discrimination learning paradigm. The order of context presentation is reversed on day 7 of testing. b) Freezing behavior of both treatment groups over the duration of the experiment shows that TAM treated mice distinguish between contexts more consistently than vehicle treated mice. There were significant interactions between day and context for each treatment group (Vehicle $p<0.0001$, TAM $p=0.0002$). Days in which freezing differs significantly between contexts are indicated. *$p<0.05$. Results are presented as mean ± SEM.

We next tested a separate cohort of mice on a more difficult version of this pattern separation task in which the order of contexts was changed throughout the experiment in a pseudo-randomized fashion (Figure 2.8 a). Analysis of freezing behavior over days showed that

TAM treated mice displayed different levels of freezing between the two contexts by Day 5, four days before control mice, suggesting that mice with increased neurogenesis have improved pattern separation (Figure 2.8 b).

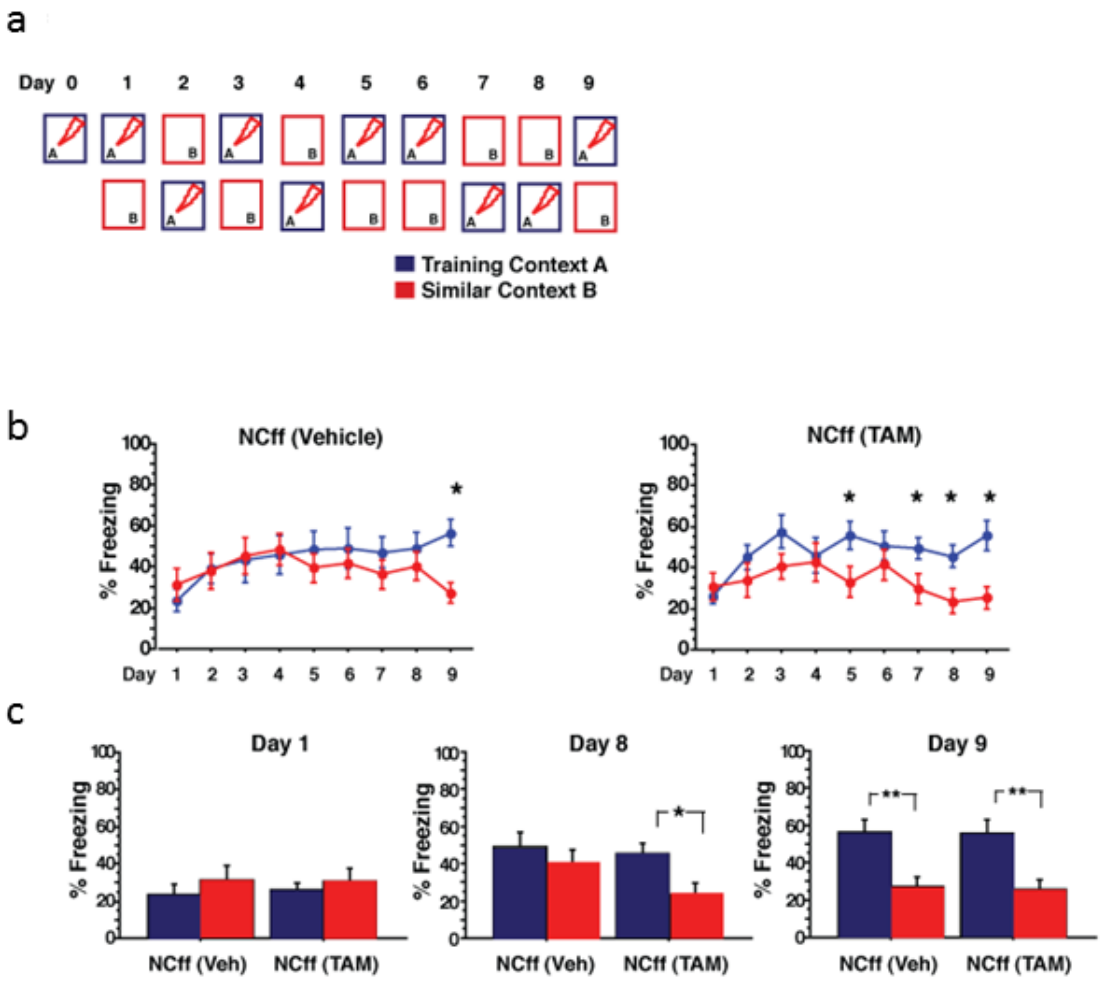

Figure 2.8 Increasing adult hippocampal neurogenesis improves performance in a fear discrimination learning paradigm with pseudo-randomized order of context presentation.
a) Contextual fear discrimination learning paradigm. The order of context presentation was pseudo-randomized. b) Freezing behavior of both treatment groups over the duration of the experiment. Both vehicle and TAM treated mice show significant differences in freezing levels between contexts over days (Vehicle: p=0.004, TAM: p=0.0008). TAM treated mice display different levels of freezing between contexts A and B by Day 5, while Vehicle animals do not display this difference until Day 9. c) Freezing behavior of both treatment groups on days 1, 8 and 9 show that TAM treated mice display different levels of freezing between contexts before vehicle treated mice. n=9-11/group. * p<0.05, **p<0.01. Results are presented as mean ± SEM.

In a separate cohort of mice, we found that TAM and vehicle treated mice show similar extinction and reinstatement to learned fear (Figure 2.9), suggesting that the difference in fear discrimination learning is not due to differences in either of these processes.

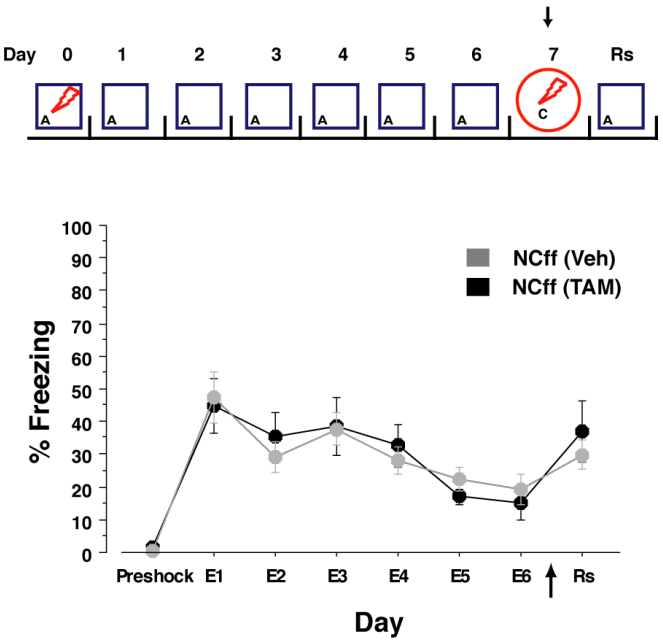

Figure 2.9 TAM and Vehicle treated animals show similar extinction and reinstatement of learned contextual fear.
Experimental design of extinction and reinstatement (Rs) learning paradigm (top). Both vehicle and TAM treated groups show similar freezing behavior during extinction learning and reinstatement (bottom). Results are presented as mean ± SEM.

2.3.3 Increasing adult hippocampal neurogenesis has no effect on mood-related behavior.

Many environmental conditions that increase neurogenesis also impact mood-related behavior, including exercise, environmental enrichment and antidepressants. We therefore wondered whether specifically increasing adult hippocampal neurogenesis, without the other effects of these environmental manipulations, would be sufficient to impact mood-related behavior. In order to test this, a cohort of *iBax* mice was administered either TAM or vehicle,

and behavioral testing started six weeks later (Figure 2.10 a). No significant differences were seen between treatment groups in the open field, elevated plus maze, tail suspension or forced swim tests (Figure 2.10 b-e).

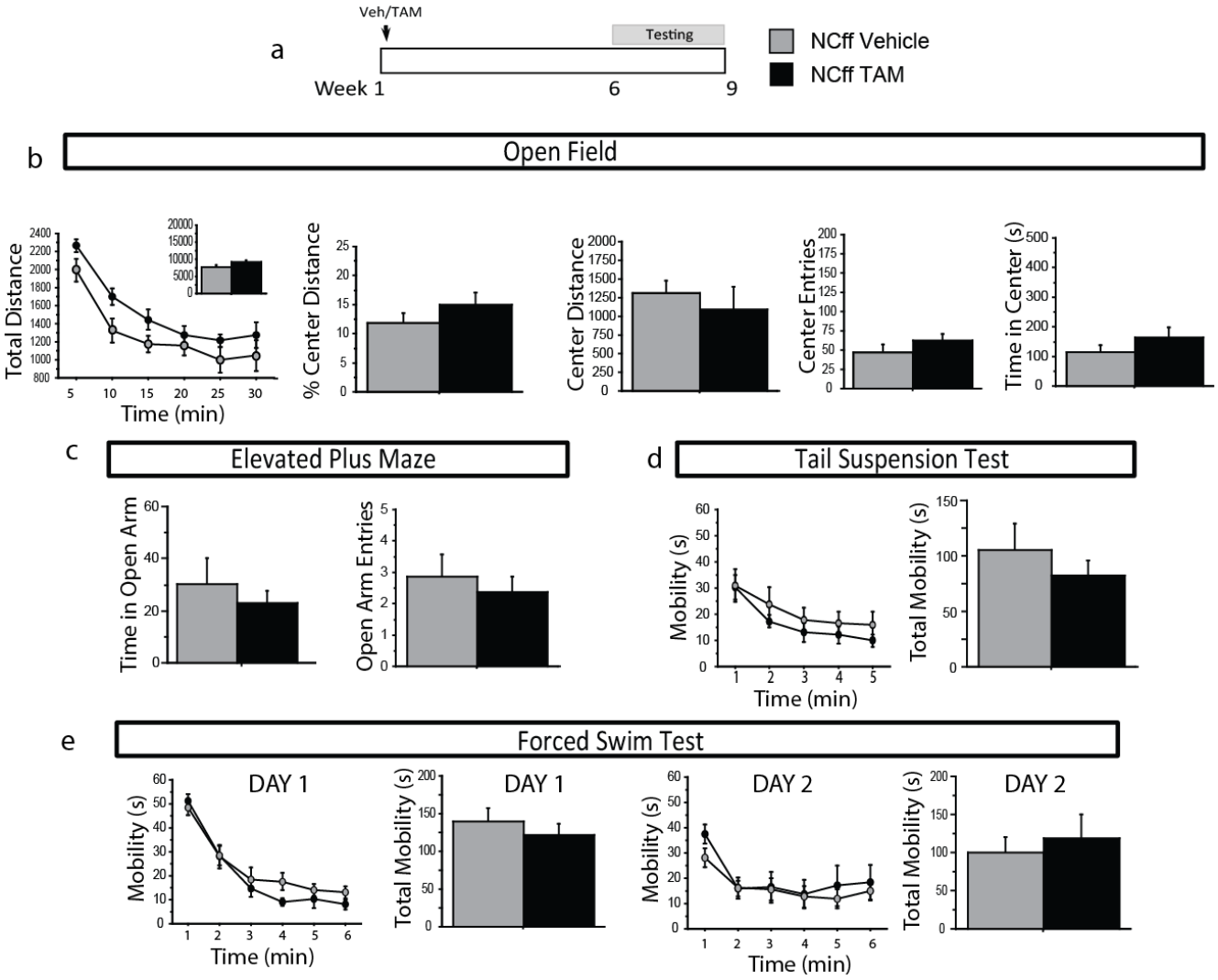

Figure 2.10 Increasing adult hippocampal neurogenesis has no effect on anxiety or depression-related behavior.

a) Experimental design. b) There were no statistically significant differences between groups in the open field test for total distance, percent center distance, total center distance, center entries or time in center (all $p>0.05$). c) There was no effect of TAM in the elevated plus maze for time in open arms and open arm entries ($p>0.05$). d) There was no effect of TAM in the tail suspension test for total mobility ($p>0.05$). e) There was no effect of TAM in mobility on either day 1 or day 2 of the forced swim test ($p>0.05$). n=8/group. Results are presented as mean ± SEM.

Neurogenesis has been hypothesized to impact behavior by affecting HPA axis regulation, since ablation of adult hippocampal neurogenesis has been shown to impair this process (Schloesser et al. 2009, Snyder et al. 2011, Surget et al. 2011). We therefore sought to determine whether increasing adult hippocampal neurogenesis would affect HPA axis regulation in response to acute stress. In order to test this, we used two paradigms that have been shown to be affected by ablation of neurogenesis. We found that increasing adult hippocampal neurogenesis in *iBax* mice has no effect on plasma CORT levels in response to thirty minutes of restraint stress, or fifteen minutes in a novel cage, a milder stressor (Figure 2.11). These data suggest that increasing adult hippocampal neurogenesis on its own is not sufficient to affect behavior or HPA axis regulation at baseline. It appears that while neurogenesis is necessary for proper HPA axis regulation, increasing neurogenesis above control levels does not have an additive effect.

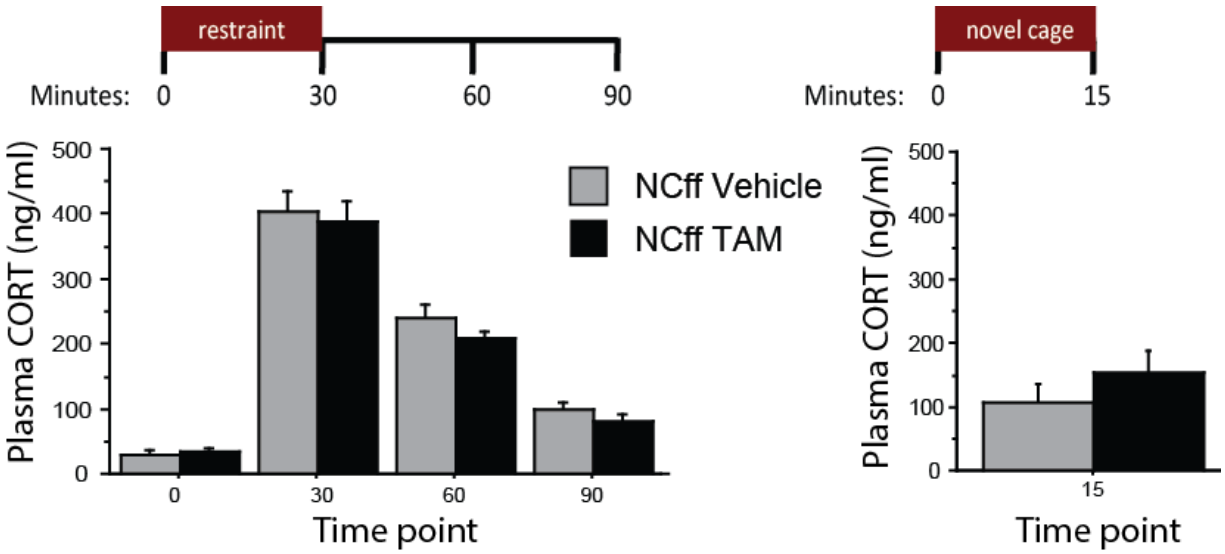

Figure 2.11 Increasing adult hippocampal neurogenesis has no effect on HPA-axis regulation
There are no differences in plasma CORT levels between TAM and vehicle treated *iBax* mice at baseline (Left, time point 0), following restraint stress (left, time points 30, 60 and 90) or following the mild stress of placement in a novel cage (right) (all $p>0.05$). n=4-11/group/time point. Results are presented as mean ± SEM.

2.3.4 Increasing adult hippocampal neurogenesis in mice exposed to voluntary exercise increases exploratory behavior.

Voluntary exercise is an environmental manipulation known to increase adult hippocampal neurogenesis (van Praag et al. 1999), and to increase dendritic complexity and spine density of dentate granule cells (Eadie et al. 2005). We therefore wished to determine whether these conditions would enhance the contribution of increased levels of adult hippocampal neurogenesis to behavior. To do this, female *iBax* mice were placed in large cages with running wheels. Under these conditions, TAM treated animals displayed increased levels of neurogenesis, with a modest effect on the number of DCX-positive neurons ($p=0.05$) and a large increase in the number of BrdU-positive cells (4.4 fold increase, $p<0.0001$) (Figure 2.12 b). Notably, there was no difference in the percent of BrdU-labeled cells that were colabeled with the neuronal marker NeuN (Figure 2.12 c).

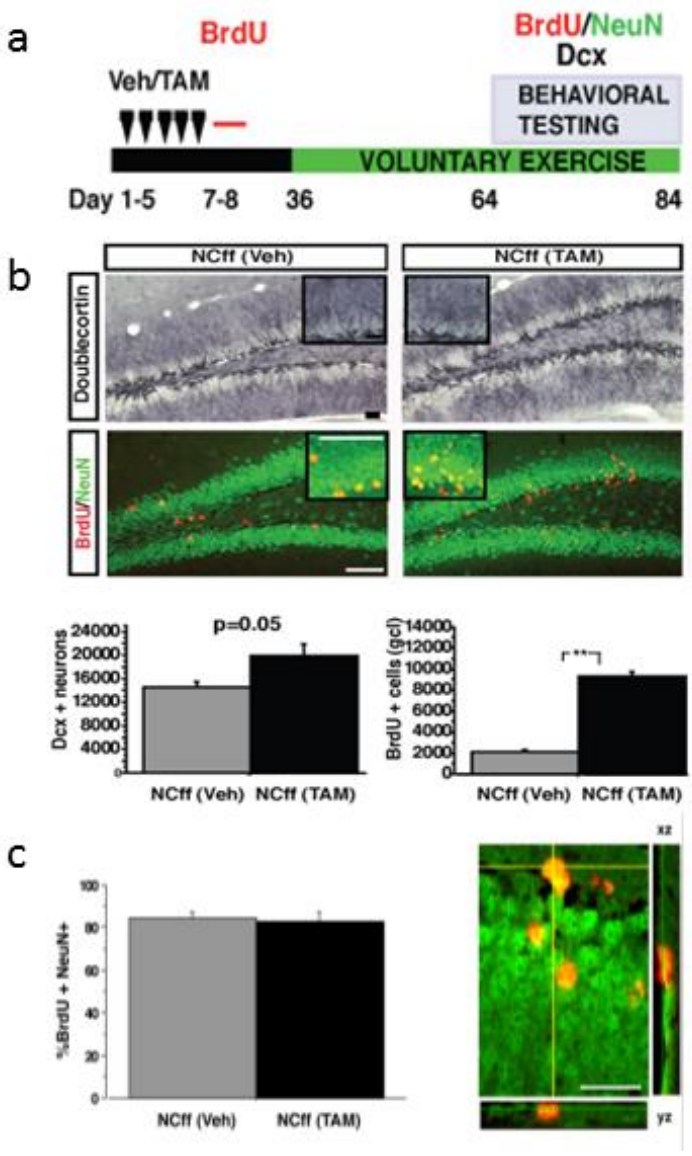

Figure 2.12 TAM increases neurogenesis in *iBax* mice exposed to voluntary exercise.
a) Experimental design. Mice were injected with vehicle or TAM. Voluntary exercise began 5 weeks later, and behavioral testing began after another 4 weeks. BrdU was injected over 2 days, 11 weeks before sacrifice. b) Representative images of DCX and BrdU immunostaining with higher magnification insets (Scale bar 100 µm). TAM treated animals show a modest increase over vehicle treated animals in total levels of DCX-positive neurons (14,527±987 (vehicle), 19,893±2,022 (TAM) (p=0.5)). (Note that analysis of DCX-positive neurons with tertiary dendrites was not feasible due to the high number of overlapping DCX-positive dendritic trees.) TAM treated animals showed a large increase in BrdU-positive cells compared to vehicle treated animals (2,119±204 (vehicle), 9,324±463 (TAM) (p<0.0001)). c) There was no difference between groups in the percent of BrdU-positive cells that were also NeuN-positive (84.2±2.3% (vehicle), 83±3.7% (TAM), (p>0.05)). Scale bar 50µm. n=4-5 mice/group for all analyses presented. **p<0.01. Results are presented as mean ± SEM.

Mice were tested in a variety of behavioral assays for exploratory, anxiety and depression-related behavior. We observed that TAM treated mice displayed increased exploratory and decreased anxiety-like behavior in the open field test, as demonstrated by increased total path length, rearing, percent pathlength center and time in center (all $p<0.05$) (Figure 2.13 b). However, we observed no differences in anxiety and depression-like behavior between groups in the light/dark test, novelty suppressed feeding, or forced swim tests ($p>0.05$) (Figure 2.14). This suggests to us that differences in open field behavior are likely due to increased exploration rather than an effect on anxiety. Notably, we also observed no difference in home cage activity (Figure 2.14), suggesting that the increased exploration in the open field test is novelty induced.

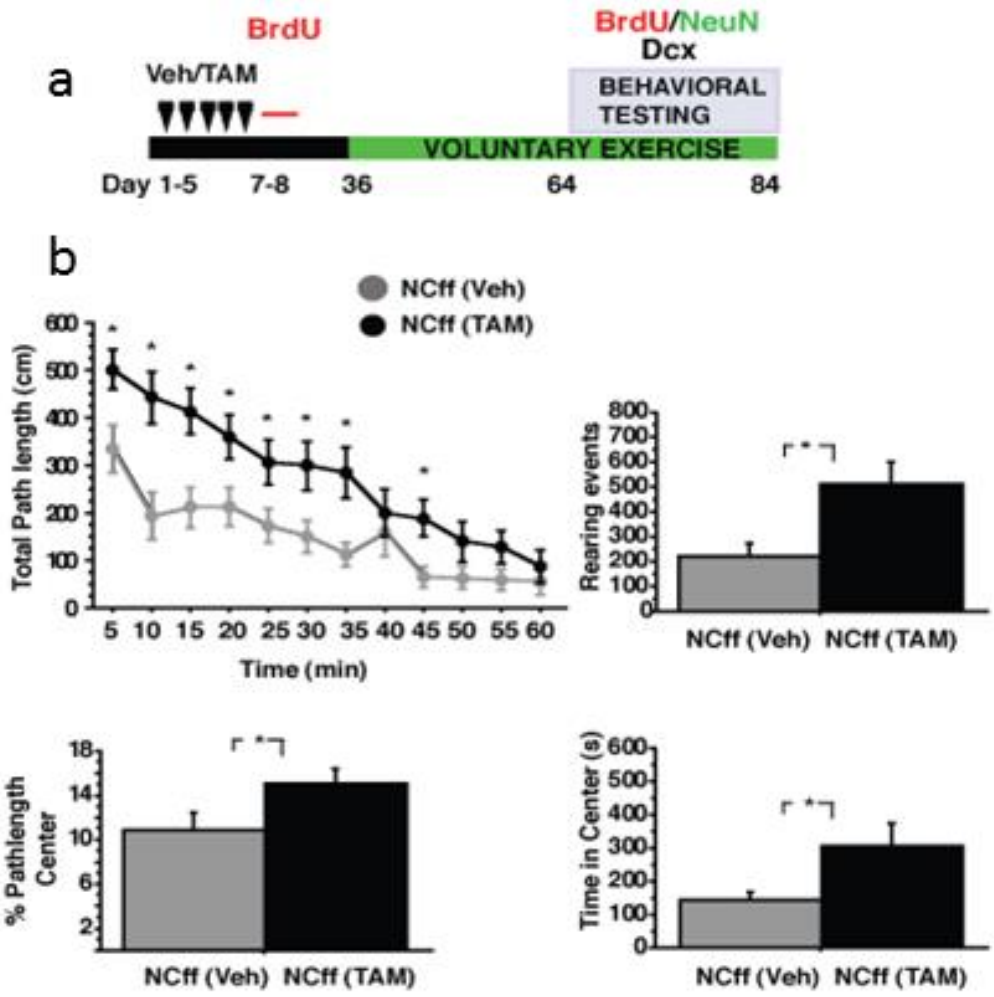

Figure 2.13 TAM treatment in *iBax* mice exposed to voluntary exercise increases exploratory behavior. a) Experimental Design. b) TAM mice showed significantly increased locomotor activity over time, as well as increased rearing events, percent pathlength center and time in center in the open field test compared to vehicle treated mice. n=10-11 mice/group. *p<0.05, **p<0.01. Results are presented as mean ± SEM.

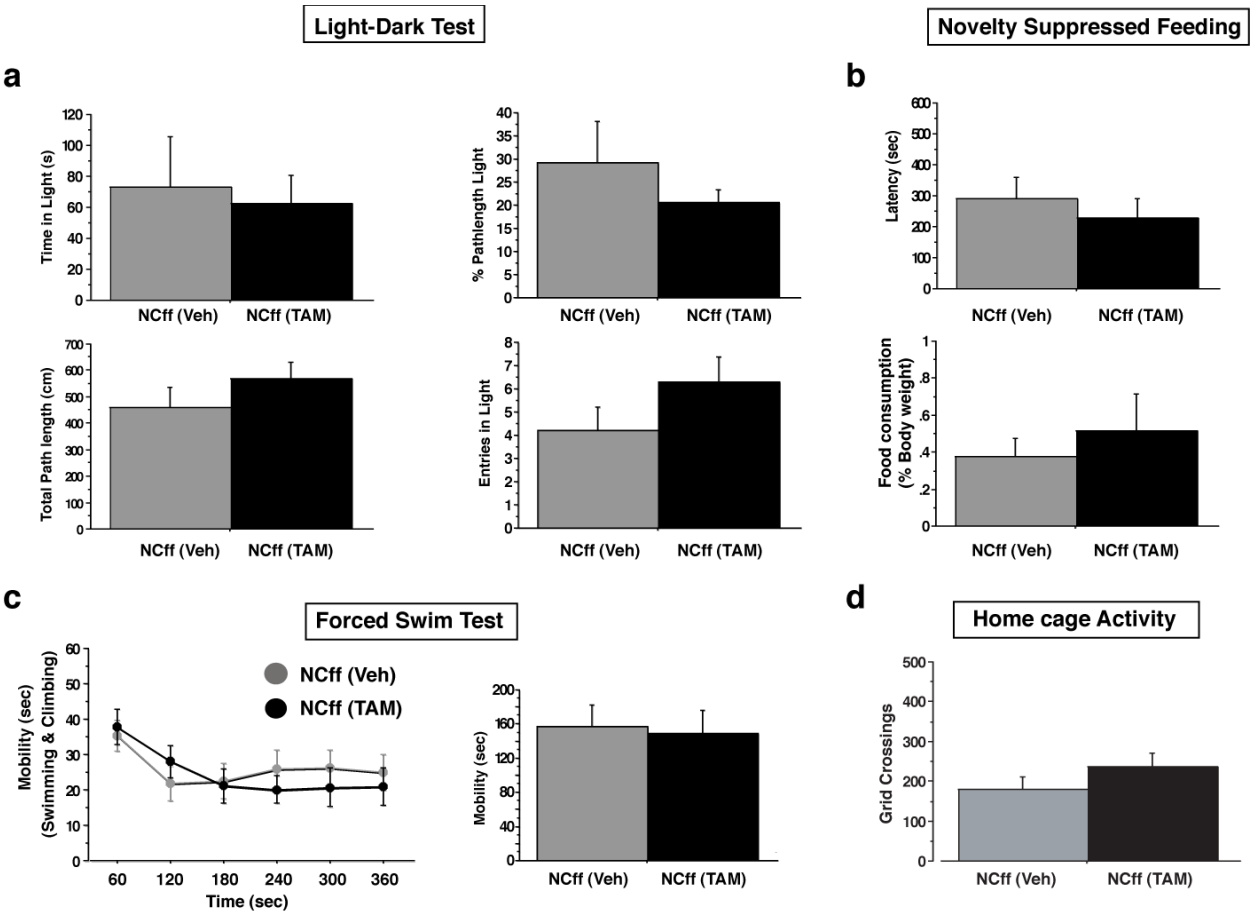

Figure 2.14 TAM treatment in *iBax* mice exposed to voluntary exercise does not affect mood-related behavior.
Following voluntary exercise, *iBax* mice and controls exhibit similar anxiety and depression-related behavior. (a) Groups behave similarly in the Light-Dark test. (b) There is no difference between groups in latency to feed or home cage food consumption in the Novelty Suppressed Feeding paradigm. c) There is no difference between groups in mobility on day 2 of the Forced Swim test. d) Home cage activity following voluntary exercise is similar between groups, as assessed by average number of grid crossings in the home cage. For a-c, n=10-11 mice/group. For d, n=7 mice/group. Results are presented as mean ± SEM.

2.5 Discussion

Here we characterize a transgenic mouse line used to increase the number of adult-born neurons. We show that this expansion is sufficient to improve behavior in contextual fear discrimination learning paradigms, suggesting enhanced pattern separation; however, it is not sufficient to affect mood-related behavior or HPA-axis regulation under baseline conditions.

When combined with voluntary exercise, genetic expansion of adult hippocampal neurogenesis is sufficient to increase exploratory behavior in the open field test, but still does not affect anxiety and depression-related behavior in other tests.

2.5.1 Characterization of *iBax* mice

iBax mice provide a genetic mouse model to increase adult hippocampal neurogenesis. Since neurogenesis is increased by rescuing adult-born cells from cell death, we expect to see differences in levels of neurogenesis when assessed after the cell death phase. Supporting this expectation, we see no difference in proliferation, as assessed by the number of cells expressing Ki67 (Figure 2.2 f). Since the cell death phase overlaps with the timing of DCX expression (Figure 2.1), we expected and observed an effect of TAM on the total number of DCX-positive cells, and a larger effect on the number of DCX-positive cells with tertiary dendrites, the more mature subset of DCX- expressing cells, most of which are beyond the cell death phase. Furthermore, we see even larger effects using BrdU to look at survival of adult-born cells. When BrdU is injected, it becomes incorporated into dividing cells and at a later time point, BrdU therefore labels cells that were undergoing division at the time of BrdU injection, as well as additional progeny of those cells. BrdU therefore amplifies the observed increase in neurogenesis by labeling cells born at various time points, all of which have increased survival.

2.5.2 *iBax* mice and pattern separation

Hippocampal-dependent contextual fear conditioning has been shown to be impaired by ablation of adult hippocampal neurogenesis in some studies (Saxe et al. 2006, Winocur et al. 2006, Imayoshi et al. 2008), but not in others (Shors et al. 2002, Clark et al. 2008). A study from

the Hen lab has shown that it is specifically in fear conditioning tasks with little training (one trial, one shock), where adult hippocampal neurogenesis affects learning, while there is no effect in paradigms with more extensive training (Drew et al. 2010). Here we do not see any effect of ablation or increase of adult hippocampal neurogenesis on contextual fear conditioning, using a minimal training paradigm (Figures 2.5 c and 2.6); however, mice of different strains respond differently to fear conditioning, therefore it is possible that increasing adult hippocampal neurogenesis in this mixed strain could impact contextual fear conditioning under a different paradigm, or that increasing adult hippocampal neurogenesis in a different strain might affect performance in this task.

We found that ablation of adult hippocampal neurogenesis impairs behavior in a fear discrimination learning task (Figure 2.5), supporting other findings that neurogenesis is required for normal pattern separation (Nakashiba et al. 2012, Tronel et al. 2012). Using *iBax* mice, we then show that increasing adult hippocampal neurogenesis is sufficient to improve behavior, especially in a more difficult version of this task where the daily order of context presentation varies (Figures 2.7 and 2.8). In these experiments, it is unclear whether increased adult hippocampal neurogenesis improves performance over controls when context presentation order is varied due to a specific role for adult-born cells in utilization of temporal cues. Alternatively, adult-born cells may play a non-specific role that only becomes relevant in more difficult pattern separation tasks, regardless of modality. Future experiments could be conducted to vary other modalities, such as scent or lighting, to make the contexts more similar in order to determine whether adult hippocampal neurogenesis plays a role in contextual fear discrimination learning paradigms made more difficult in these ways. Another outstanding question is whether increasing adult hippocampal neurogenesis can improve pattern separation in additional tasks

that do not involve contextual fear learning, for example radial arm maze and touch screen paradigms that require fine spatial discrimination in which performance is impaired in mice with ablated adult hippocampal neurogenesis (Clelland et al. 2009). In additional experiments not shown here, increasing neurogenesis in *iBax* mice had no effect on object recognition of similar objects in the novel object test, on spatial learning and memory in the reference version of the Morris water maze, nor on reversal learning in the active place avoidance task (Sahay et al. 2011). However additional testing is required to determine the full extent of pattern separation tasks that may be affected by increasing levels of adult hippocampal neurogenesis.

It is worth noting that in the adult brain, the Nestin promoter governs expression in neural stem cells in both the SGZ and SVZ, and therefore *Bax* is knocked out of adult born cells in both of these regions in *iBax* mice. In fact, we do see increased neurogenesis in both the hippocampus and the olfactory bulb, as observed following BrdU injections (Figures 2.2 and 2.3). The fact that the dentate gyrus has been implicated as playing a role in pattern separation, and that x-irradiation specifically of the hippocampus impairs performance in this task (Figure 2.5), suggests that the increase in adult *hippocampal* neurogenesis underlies enhanced performance in the pattern separation task described here. However it is possible that the addition of new neurons from the SVZ to the olfactory bulb could be involved in this task as well. Young, adult-born granule cells in the olfactory bulb have been shown to be more responsive to novel odors (Magavi et al. 2005), and to be involved in odor discrimination (Moreno et al. 2009). However, a study which blocked cell death specifically in the olfactory bulb, thereby increasing the number of surviving adult-born cells in this region, found that this manipulation impaired performance in an odor discrimination task (Mouret et al. 2009). Therefore it is unclear whether increased

survival of adult-born cells in the olfactory bulb affects behavior in the fear discrimination learning task.

In order to determine whether neurogenesis in the SVZ is involved in this task, focal x-irradiation could be delivered selectively to the SVZ in *iBax* mice with increased neurogenesis. If ablation of neurogenesis in the SVZ does not prevent the enhanced pattern separation in TAM treated *iBax* mice, this would suggest that neurogenesis in the SVZ is not involved in this task. Alternatively, experiments could be designed to specifically increase adult hippocampal neurogenesis without affecting the olfactory bulb through viral delivery of Cre to the dentate gyrus of mice homozygous for the floxed *Bax* allele. Additionally, an experiment such as this could deliver virus specifically to the dorsal or ventral halves of the dentate gyrus in order to test the hypothesis that increasing neurogenesis in the dorsal, but not ventral dentate gyrus would be sufficient for the enhanced pattern separation observed here. This hypothesis stems from the recent finding that ablation of adult hippocampal neurogenesis in the dorsal, but not ventral, hippocampus impairs pattern separation (Wu and Hen 2014).

2.5.3 Increasing adult hippocampal neurogenesis does not affect anxiety or depression-related behavior, or HPA axis regulation under baseline conditions

Since various studies in which adult hippocampal neurogenesis is ablated have shown that neurogenesis is required for some of the behavioral effects of antidepressants (Santarelli et al. 2003, David et al. 2009), we hypothesized that increasing adult hippocampal neurogenesis might also be sufficient to elicit similar behavioral effects as antidepressants. However, under baseline conditions, we observed no effect of increasing adult hippocampal neurogenesis on anxiety and depression-related behavior (Figure 2.10). Although the lack of an effect on anxiety

and depression-related behavior initially suggested to us that increasing adult hippocampal neurogenesis is not sufficient for the behavioral effects of antidepressants, we also note that antidepressants often do not have effects under baseline conditions, but rather only affect behavior in stressed animals (David et al. 2009, Surget et al. 2009). Experiments testing the effects of increasing adult hippocampal neurogenesis in stressed animals will be presented in Chapter 3.

Interestingly, we also do not see any effect of increasing adult hippocampal neurogenesis on HPA axis activity at baseline or in response to acute stress (Figure 2.11). Since studies have shown that ablation of adult hippocampal neurogenesis impairs regulation of the HPA axis (Schloesser et al. 2009, Snyder et al. 2011, Surget et al. 2011), we expected that increasing adult hippocampal neurogenesis might improve HPA axis regulation, which we expected to observe as decreased plasma CORT following acute stress. However, our data suggest that increasing adult hippocampal neurogenesis does not affect this process. It is relevant to note that glucocorticoids, and signaling through their receptors, are involved in many cellular processes, and therefore that maintaining normal HPA axis regulation in the presence of increased adult hippocampal neurogenesis may in fact be adaptive.

2.5.4 An increase in adult hippocampal neurogenesis combined with voluntary exercise increases exploration.

We find that in *iBax* mice exposed to voluntary exercise, mice with a genetic expansion of adult hippocampal neurogenesis display increased exploratory behavior in the open field test (Figure 2.13). This finding further supports an emerging role for the dentate gyrus, and the hippocampus as a whole, in mediating exploratory behavior.

A role for the hippocampus in motivated behavior, such as exploration and foraging, has been suggested (Swanson 2000), although the evidence for this role is inconsistent. Increased exploration in the open field has been observed following lesion of the hippocampus (Jarrard and Bunnell 1968, Rossi-Arnaud and Ammassari-Teule 1992, Ammassari-Teule and Passino 1997), although this effect is not always observed (Markowska and Lukaszewska 1981), and recent optogenetic studies from our lab have implicated the dentate gyrus in playing a role in exploratory behavior, in that stimulation of dorsal dentate granule cells increases exploration (Kheirbek et al. 2013). Here, we find that increasing adult hippocampal neurogenesis in mice exposed to voluntary exercise also increases exploration (Figure 2.13). All of these results together suggest that shifting hippocampal activity in many different ways may lead to increased exploration.

Interestingly, we observed an effect of increasing adult hippocampal neurogenesis on exploratory behavior, but only in mice exposed to voluntary exercise. Although it is difficult to precisely compare the effects of TAM on levels of neurogenesis between mice at baseline and voluntary exercise due to different experimental designs, it appears that the increases in neurogenesis are similar (3.6 fold increase in BrdU at baseline 8 weeks post injection compared to a 4.4 fold increase in BrdU in mice exposed to voluntary exercise 11 weeks post injection). Therefore, we suggest that the observed exploratory effect in mice exposed to voluntary exercise is not due solely to the increase in neurogenesis, but rather that exercise modifies the properties of the increased numbers of excitable adult-born neurons in a way that impacts exploratory behavior. In the dentate gyrus, exercise has been shown to enhance LTP (van Praag et al. 1999), increase expression of BDNF, as well as the NR2B subunit of the NMDA receptor (Farmer et al. 2004), and to increase spine density (Eadie et al. 2005). These effects of exercise likely alter

incorporation of adult-born granule cells into hippocampal circuits in such a way that increasing the number of adult-born neurons in the presence of voluntary exercise may affect circuits differently than at baseline.

Together, the data presented here suggest that increasing adult hippocampal neurogenesis is sufficient to enhance certain cognitive processes such as pattern separation, providing a potential therapeutic target for impairments in this process, for example as observed during aging (Yassa et al. 2011) and during post-traumatic stress disorder (Lissek et al. 2010). Furthermore, we observed that increasing adult hippocampal neurogenesis in mice subjected to voluntary exercise increases exploration, while this effect was not seen under baseline conditions. This disparity suggests that levels of adult hippocampal neurogenesis can affect distinct behaviors when combined with different environmental conditions. This dissociation, along with potential downstream circuits that may mediate the behaviors sensitive to changes in adult hippocampal neurogenesis, will be further discussed in Chapter 4.

2.6 Involvement

The work presented in this chapter was conducted in collaboration with many members of the Hen Lab. I was specifically involved in immunohistological characterization and behavioral testing of *iBax* mice, contributing to some of the experiments performed at baseline and all of the experiments performed following voluntary exercise.

Chapter 3: Increasing adult hippocampal neurogenesis is sufficient to reduce anxiety and depression-like behaviors.

3.1 Introduction

As previously discussed in more detail (Chapter 1), adult hippocampal neurogenesis is a process through which additional granule cells are added to the dentate gyrus throughout life. These cells are produced from progenitors located in the subgranular zone of the dentate gyrus, and their rates of proliferation, maturation and survival are impacted by environmental conditions such as age, stress, exercise and antidepressants (Gould et al. 1997, Malberg et al. 2000, van Praag et al. 2005, Dranovsky et al. 2011).

Many antidepressants are known to act through monoamine systems, however the downstream mechanisms through which they affect mood are still not entirely understood. While many antidepressants change monoamine levels within hours, effective changes in mood are not seen for three to four weeks. This disparity, along with the findings that antidepressants increase the number of adult-born neurons (Malberg et al. 2000, Boldrini et al. 2009), which take about four weeks to form synaptic connections (Toni et al. 2007) and contribute to behavior in rodents (Kee et al. 2007, Denny et al. 2012), led to the hypothesis that antidepressants might affect mood by increasing adult hippocampal neurogenesis (Duman et al. 2001). Since these initial observations, adult hippocampal neurogenesis has been shown to be required for some, but not all, of the behavioral effects of antidepressants (Santarelli et al. 2003, David et al. 2009). However, one important remaining question is whether increasing levels of adult hippocampal neurogenesis is *sufficient* for antidepressant-like effects on behavior. Tangential support for this hypothesis includes a study in which deletion of neurofibromin 1 (Nf1) from adult-born cells increased levels of adult hippocampal neurogenesis and had an antidepressant-like effect on

behavior; however this manipulation not only increased the number of adult-born neurons, but also affected these cells in other ways, such as by activating ERK signaling (Li et al. 2012). More recently, the P7C3 compound has been shown to increase adult neurogenesis, and has an antidepressant-like effect on social interaction behavior following social defeat; however the mechanism by which this drug increases neurogenesis, as well as additional effects of the drug that may contribute to behavior, are unknown (Walker et al. 2014).

In order to directly assess the effects of selectively increasing the number of adult-born neurons, we used a transgenic mouse line with increased adult hippocampal neurogenesis. Under baseline conditions, increased adult neurogenesis enhances pattern separation, but does not impact anxiety or depression-related behavior (Chapter 2). Here, we show that increasing adult hippocampal neurogenesis using this same transgenic mouse model is sufficient to provide resilience to chronic corticosterone (CORT) administration, a model of anxiety and depression, but does not affect performance in a discrimination learning task in mice treated with CORT. In a separate set of experiments in wild-type mice, we find that treatment with the BAX antagonist iMac2 in mice treated with chronic CORT increases adult hippocampal neurogenesis and decreases anxiety-like behavior, but also does not affect performance in a discrimination learning task. Furthermore, we find that both genetic and pharmacological manipulations alter the proportion of adult-born cells that become neurons or oligodendrocytes specifically in the ventral dentate gyrus, lending support to the recent hypothesis that changes in adult-born cell fate and oligodendrogenesis may impact anxiety and depression-related behavior (Edgar and Sibille 2012, Chetty et al. 2014). These findings extend our understanding of the contribution of adult neurogenesis to anxiety and depression, provide insight into the role of adult-born cell fate in

mediating the effects of stress and antidepressants, and suggest that neurogenesis may be targeted in the development of novel antidepressants.

3.2 Methods

3.2.1 Mice

iBax mice are described in Section 2.2.1. Wild type C57BL/6 mice (Taconic, Germantown, NY) were used for the iMac2 experiments. All mice were 8-10 weeks old at the beginning of each experiment. Mice were housed 2-5 per cage and maintained on a 12 hour light/dark schedule with continuous access to food and water. All behavioral testing was conducted during the light cycle with approval from the Institutional Animal Care and Use Committees at both Columbia University and the New York State Psychiatric Institute.

3.2.2 Drug administration

Tamoxifen (TAM) was dissolved in a solution of corn oil (C8267, Sigma, St. Louis, MO) and 10% ethanol. 8-10 week old *iBax* mice received 2 mg TAM (10 mg/ml, Sigma), or the same volume of corn oil and ethanol (vehicle), intraperitoneally once per day for 5 consecutive days.

iMac2 (a generous gift from Inception Sciences, San Diego, CA) was dissolved to 1 mg/ml in water and administered at 10 mg/kg via daily gavage, starting when mice were 8-10 weeks of age. Control animals received an equivalent volume of water via gavage.

Bromodeoxyuridine (BrdU) (150 mg/kg body weight, dissolved in .9% NaCl; Roche, Indianapolis, IN) was injected intraperitoneally once per day for 2 days at the time points designated in the experimental timelines.

3.2.3 CORT administration

Here, we chose to model anxiety and depression using chronic CORT administration. When modeling human conditions in rodents, it is important to consider the validity of the model, which is typically assessed in three domains: face, construct and predictive validity (Willner and Mitchell 2002). Models of anxiety and depression are designed and validated based on whether they are elicited by risk factors for depression (construct validity), whether they lead to depression-like behavior (face validity), and whether they are reversed by chronic antidepressant treatment (predictive validity).

Depression and anxiety disorders have often been modeled in rodents using chronic stressors, such as restraint, social isolation or electric shocks. Chronic unpredictable stress (CUS) or chronic unpredictable mild stress (CUMS) consist of a protocol where mice are subjected to variable stressors over a period of weeks, including isolation, light/dark cycle reversal, foot shocks, restraint, sleep deprivation or a dirty home cage environment. This type of stress model has been shown to produce depression-like behavior (face validity), which can be reversed with chronic antidepressant treatment (predictive validity) (Willner 2005).

Another more recently used model of anxiety and depression is chronic administration of high levels of CORT. The use of this model is based on the finding that individuals with increased CORT levels due to pituitary or adrenal tumors (diagnosed as Cushing's disease), have an increased risk of depression (construct validity) (Sonino et al. 1998). Furthermore, some depression patients who do not have Cushing's disease display impaired HPA axis regulation (Greden et al. 1983, Vreeburg et al. 2009), suggesting that a model based on increased CORT levels may be relevant to the larger patient population. CORT has been administered over several weeks as a model of chronic stress in rodents, leading to anxious and depressed phenotypes (face

validity) as well as decreased adult hippocampal neurogenesis (Ardayfio and Kim 2006, Murray et al. 2008, Zhao et al. 2008). The effects of chronic CORT on various behavioral tests can be rescued by antidepressants, providing predictive validity (Gourley et al. 2008, David et al. 2009).

Here, CORT (C2505, Sigma) was dissolved in .45% beta-cyclodextrin, administered *ad libitum* in opaque bottles, and replaced every 3-4 days. *iBax* mice received 70 µg/ml CORT, equivalent to 10 mg/kg/day, an effective dose in this strain (data not shown). In the iMac2 experiment, C57BL/6 mice received 35 µg/ml CORT, equivalent to 5 mg/kg/day (David et al. 2009). Non-CORT treated mice received .45% beta-cyclodextrin alone.

3.2.4 Behavioral testing

Behavioral testing was performed as described in Sections 2.2.3 and 2.2.4.

3.2.5 Plasma corticosterone

To examine HPA axis response to acute stress in the chronic CORT model (Figure 2e), mice were subjected to a 1 minute swim stress, where animals were placed in a beaker of room temperature water. Trunk blood was collected 5 minutes later (as in (David et al. 2009)) and treated as described in Section 2.2.5.

3.2.6 Immunohistochemistry

Sacrifice and immunostaining for DCX and BrdU alone were conducted as described in Section 2.2.7. For triple label of BrdU, NeuN and MBP, sections were washed in TBS, placed in a solution of 50% formaldehyde and 50% 2x SSC for 2 hours at 65°C, followed by 10 mins in 2x

SSC at room temperature, 2N HCl for 30 mins at 37°C, .1M boric acid for 10 mins at room temperature, and then washed in TBST (.1% Triton). Section were blocked in TBST and 3% normal donkey serum for 1 hour and placed in primary antibodies (sheep anti-BrdU 1:200 [ab1893, abcam, Cambridge, MA], rat anti-MBP 1:100 [ab7349, abcam], mouse anti-NeuN 1:500 [MAB377, Millipore, Billerica, MA] in blocking solution) overnight at 4°C. Sections were then washed in TBST and incubated in secondary antibodies (488 donkey anti-sheep 1:500, Cy3 Donkey anti-rat 1:500, Cy5 Donkey anti-mouse 1:200 [Jackson] in PBS) for 2 hours at room temperature. Colabel analysis was conducted by scanning z-stacks of at least 20 BrdU-positive cells from both the dorsal and ventral hippocampus of each mouse on an Olympus Fluoview confocal microscope at 60x magnification.

3.2.7 Organ weights

In the iMac2 experiment, a subset of mice that were not transcardially perfused were decapitated. The spleen and thymus were immediately dissected and weighed.

3.2.8 Statistical methods

To assess effects on behavior, HPA axis regulation and neurogenesis in the iMAC2 experiments, one way ANOVA was used. Due to our *a priori* hypotheses about the interactions between increasing neurogenesis and CORT, in the *iBax* and CORT experiments, we performed planned comparisons on vehicle vs CORT groups and CORT vs TAM+CORT groups using one way ANOVAs. Statistical analysis was conducted using StatView software (SAS Institute, Cary, NC). Results were considered statistically significant if $p < 0.05$.

3.3 Results

3.3.1 In CORT treated mice, genetic deletion of *Bax* in adult neural stem cells and progeny increases neurogenesis.

It has been repeatedly shown that ablation of adult neurogenesis has no effect on anxiety- or depression-related behavior at baseline, but can prevent the enhancing effects of antidepressants and enriched environments (Santarelli et al. 2003, Schloesser et al. 2010). We therefore reasoned that even though increasing adult neurogenesis shows no effect on behavior at baseline, it might prevent the effects of stress. Furthermore, in some strains of mice, antidepressants have no effect at baseline, but do rescue the effects of chronic unpredictable mild stress (Surget et al. 2009) or chronic CORT (David et al. 2009).

First, we assessed whether genetic deletion of *Bax* from adult neural progenitors would maintain increased levels of adult hippocampal neurogenesis in mice treated with chronic CORT using the immature neuronal marker doublecortin (DCX) (Figure 3.1 b). CORT treatment significantly decreased the number of DCX-positive cells compared to vehicle (24% decrease, $F(1,7)=15.994$, $p<0.01$, Figure 3.1 d), while this was increased in the TAM+CORT group (69% increase, $F(1,10)=16.789$, $p<0.01$). We next assessed the number of DCX-positive neurons with tertiary dendrites, which represent a more mature subset of adult-born granule cells. Here, there was a trend for an effect of CORT (31% decrease, $F(1,7)=4.754$, $p=0.07$), and a significant increase in TAM+CORT treated mice (120% increase, $F(1,10)=18.025$, $p<0.01$).

Additionally, mice were injected with the thymidine-analog BrdU to assess the survival of adult-born neurons (Figure 3.1 c). While there was no significant difference between vehicle and CORT ($F(1,6)=1.086$, $p>0.05$), TAM+CORT animals had significantly greater numbers of BrdU-labelled cells compared to CORT alone (168.5% increase, $F(1,9)=5.33$, $p<0.05$, Figure 3.1

e), with similar effects in both dorsal and ventral subregions (Figure 3.2). Genetic deletion of *Bax* therefore increases survival of adult-born neurons in the presence of chronic CORT.

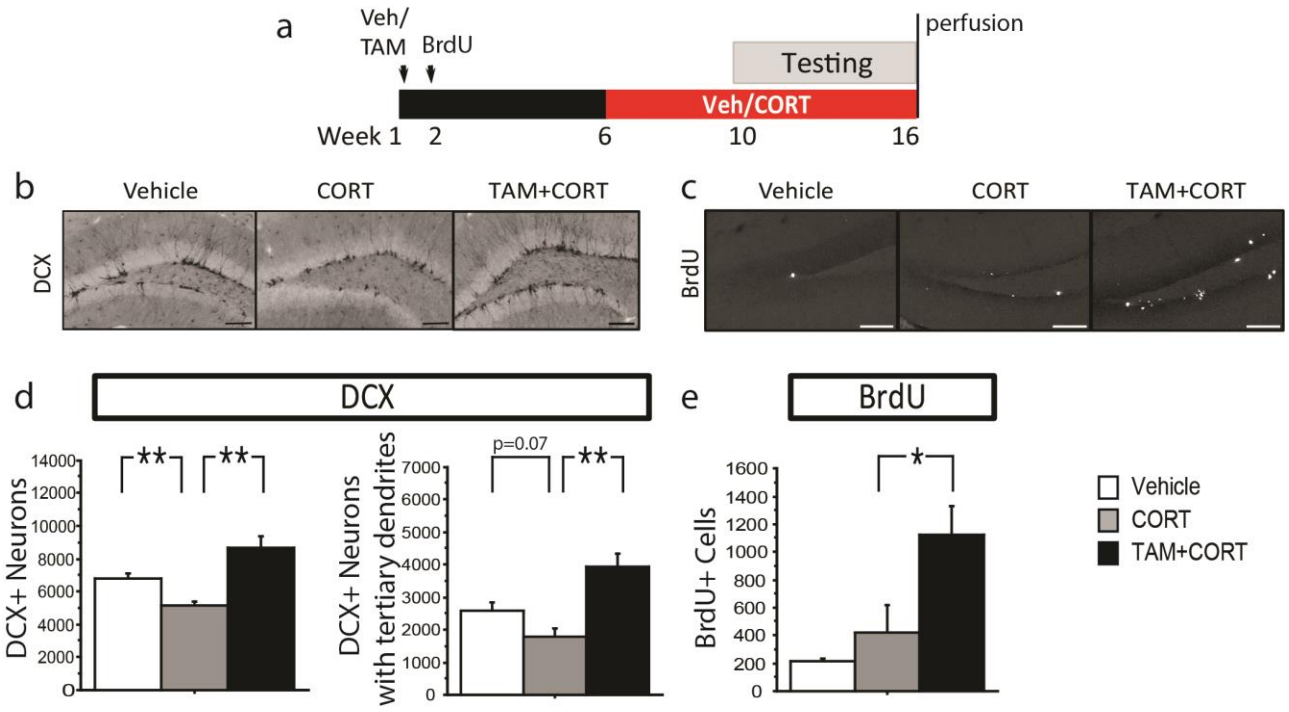

Figure 3.1 Genetic ablation of *Bax* in neural stem cells and progeny increases adult hippocampal neurogenesis in mice treated with chronic CORT.
(a) Experimental design. (b-c) Representative images of DCX and BrdU (scale bars 100 um). (d) CORT treatment decreases the total number of DCX-positive neurons (p=0.005), and there is a trend for a decrease in the number of DCX-positive neurons with tertiary dendrites (p=0.07). TAM+CORT prevents these effects (p=0.002 for total DCX-positive neurons, p=0.002 for DCX-positive neurons with tertiary dendrites). (e) TAM treatment increases the number of BrdU-positive cells (p=0.047). All error bars represent SEM. *p<0.05, ** p<0.01.

3.3.2 Increased adult hippocampal neurogenesis provides resilience to the behavioral effects of chronic CORT administration, but does not affect HPA axis regulation.

After 4 weeks of CORT administration, mice were tested on various measures of anxiety and depression-related behavior. Only a subset of these tests (elevated plus maze and tail suspension test) were affected by CORT, but interestingly, these same tests were affected by an

increase in neurogenesis (TAM+CORT group). In the elevated plus maze, CORT had an anxiogenic effect, manifested by decreased time spent in the open arms ($F(1,25)=6.86$, $p<0.05$) as well as fewer open arm entries ($F(1,25)=6.547$, $p<0.05$, Figure 3.2 b). This was reversed in TAM+CORT treated mice ($F(1,28)=6.273$, $p<0.05$ for open arm time, $F(1,28)=4.252$, $p<0.05$ for open arm entries), showing that increasing adult hippocampal neurogenesis is sufficient to produce an anxiolytic effect in this test. In the tail suspension test, CORT decreased mobility as compared to vehicle ($F(1,26)=5.13$, $p<0.05$, Figure 3.2 c), and TAM+CORT treatment increased mobility ($F(1,26)=9.597$, $p<0.01$), providing evidence that increased adult hippocampal neurogenesis is sufficient to provide stress resilience in this test of depression-related activity.

Interestingly, in the open field test, where there is no effect of CORT for either total distance traveled ($F(1,17)=1.162$, $p>0.05$) or percent center distance ($F(1,17)=1.754$, $p>0.05$), we observed no effect of TAM (total distance: $F(1,16)=0.032$, $p>0.05$, percent center distance: $F(1,16)=1.554$, $p>0.05$, Figure 3.2 a). We also observed no effects of CORT or TAM+CORT in the light/dark, forced swim or novelty suppressed feeding tests (not shown). Consistent with the lack of an effect of increasing adult neurogenesis in the baseline, no-stress group, this suggests that neurogenesis specifically affects behaviors that are impacted by chronic CORT.

Next, we were interested in assessing the effect of increasing adult hippocampal neurogenesis on HPA axis regulation. Ablation of neurogenesis has been shown to impair HPA axis regulation, which has been hypothesized as the mechanism through which changes in neurogenesis may affect anxiety and depression-related behavior (Schloesser et al. 2009, Snyder et al. 2011, Surget et al. 2011). Furthermore, previous work has shown that chronic administration of exogenous CORT alters HPA axis regulation by providing constant negative feedback to the HPA axis, and thereby preventing release of endogenous CORT from the adrenal

glands. In mice treated with chronic CORT, the adrenal glands shrink, and mice are unable to launch a normal, endogenous CORT response to acute stress (Murray et al. 2008).

In order to determine whether increasing adult hippocampal neurogenesis prevents the effects of chronic CORT on HPA axis regulation, mice were subjected to acute swim stress, a paradigm in which we have shown that animals treated with chronic CORT do not display the stress-induced increase in plasma CORT that is seen in controls (David et al. 2009). Here, we found a strong trend for a blunted response to this acute stressor in CORT treated mice compared to controls ($F(1,8)=5.155$, $p=0.05$, Figure 3.2 d), and no difference between CORT and TAM+CORT treated mice ($F(1,9)=0.016$, $p>0.05$), suggesting that both of these groups have similar impairment of the endogenous HPA axis response to acute stress. In the context of chronic CORT, there is therefore a dissociation, where increased neurogenesis affects anxiety- and depression-related behavior, but not HPA axis regulation.

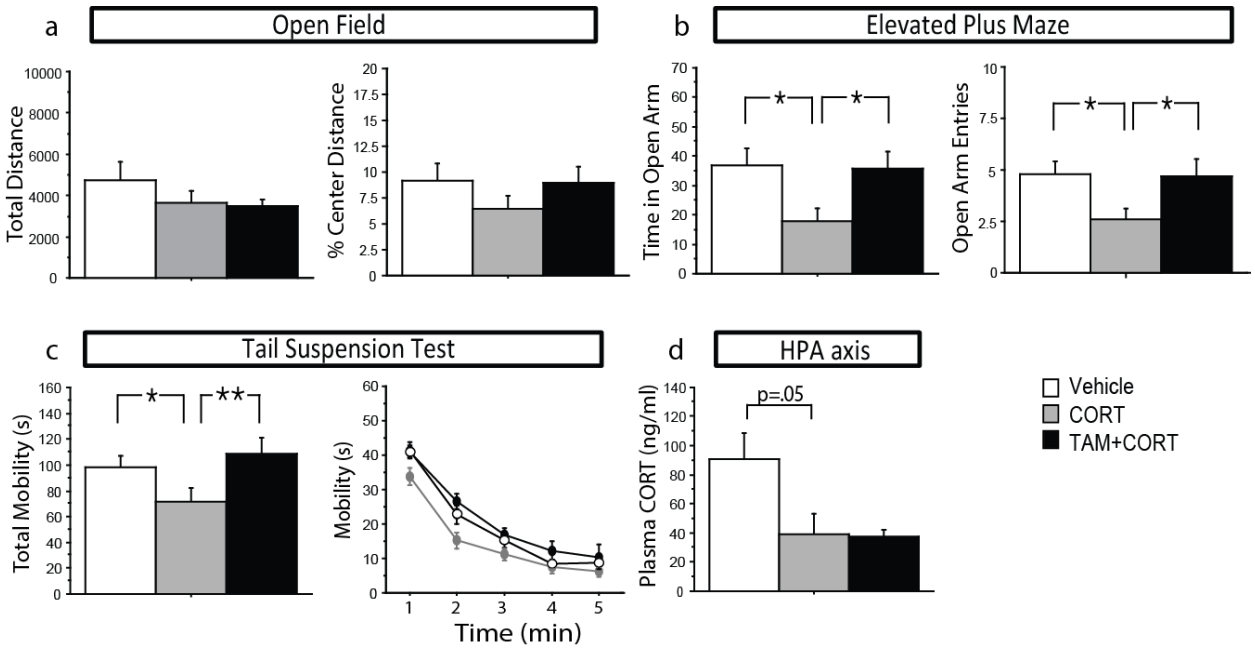

Figure 3.2 Genetically increasing adult hippocampal neurogenesis in *iBax* mice prevents the effects of chronic CORT on mood-related behavior, but not HPA axis regulation.
(a) No statistically significant differences were seen between groups in total distance or percent center distance in the open field test ($p>0.05$). n=8-10/group. (b) In the elevated plus maze, CORT treated mice spent significantly less time in the open arms ($p=0.015$) and had fewer open arm entries ($p=0.017$) than controls. These effects were reversed in TAM+CORT treated mice ($p=0.018$ for open arm time, $p=0.049$ for open arm entries). n=12-15/group. (c) In the tail suspension test, CORT treated mice displayed decreased mobility ($p=0.032$), which was reversed in TAM+CORT treated mice ($p=0.005$). In the line graph, data is represented in 1 minute bins for the duration of the test. n=14-15/group. (d) There is a strong trend for CORT treated mice to have lower plasma CORT levels than controls following forced swim stress ($p=0.05$), but no difference between CORT and TAM+CORT groups ($p=0.91$). n=5-6 group. All error bars represent SEM. *$p<0.05$, ** $p<0.01$.

3.3.3 Increased adult hippocampal neurogenesis does not affect behavior in a contextual discrimination task in mice treated with chronic CORT.

Next, we tested whether increasing adult hippocampal neurogenesis in mice treated with chronic CORT would improve behavior in cognitive-based tasks. First, mice were tested in contextual fear conditioning (Figure 3.3 a). Here, we saw no differences between groups in levels of freezing before fear conditioning (Day 1 Context A: all $F<0.170$, $p>0.05$), or after fear conditioning in either the shock context (Day 2 Context A: all $F<0.514$, all $p>0.05$), or in a novel

context (Day 2 Context C: all $F<0.857$, all $p>0.05$) (Figure 3.3 b). Furthermore, on day 2, all groups froze significantly more in context A than in context C, suggesting that they could discriminate between these two different contexts (Vehicle $F(1,8)=30.479$, $p<0.001$; CORT $F(1,8)=17.052$, $p<0.01$; TAM+CORT $F(1,12)=62.389$, $p<0.0001$).

Next, mice were tested in a fear discrimination learning paradigm, where they were exposed daily to the shock context A and the similar no shock context B, presented in a pseudo-randomized order (Figure 3.3 a). In this test, no treatment groups displayed significantly different freezing levels between Contexts A and B by Day 10 of this task (Vehicle $F(1,8)=2.143$, $p>0.05$; CORT $F(1,8)=0.088$, $p>0.05$; TAM+CORT $F(1,12)=0.023$, $p>0.05$), although vehicle mice appear to be closer to discriminating than the other groups (Figure 3.3 c, d). Increasing adult hippocampal neurogenesis did not improve behavior in this fear discrimination learning paradigm.

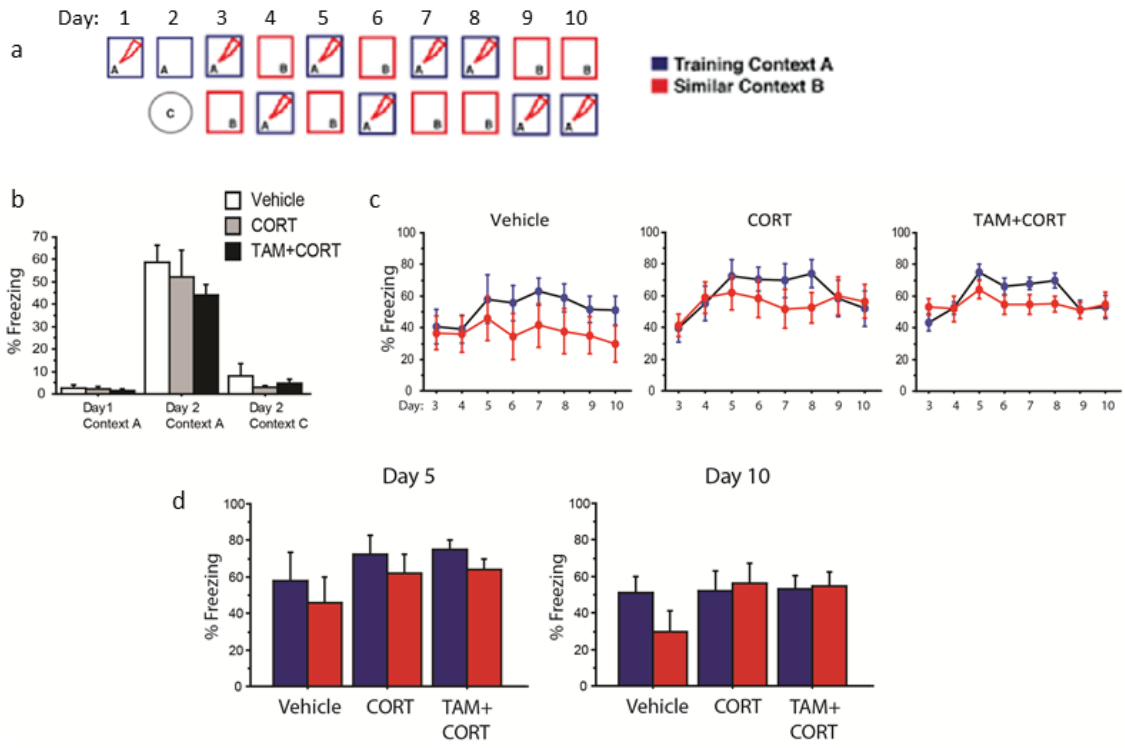

Figure 3.3 Genetically increasing adult hippocampal neurogenesis is not sufficient to affect freezing levels in a contextual discrimination learning task in mice treated with chronic CORT.
a) Experimental design. On days 1 and 2, mice were tested in contextual fear conditioning. Discrimination learning began on Day 3. The daily order of context presentation was pseudo-randomized. b) There were no differences between groups in freezing levels before conditioning (Day 1 Context A), after conditioning in the shock context (Day 2 Context A), or in a novel context (Day 2 Context C). c) Freezing levels in contexts A and B over days during the discrimination learning task. d) Freezing levels in both contexts on Days 5 and 10. There is no difference between freezing levels in mice treated with CORT alone, or treated with TAM+CORT. n=5-7/group. Results are presented as mean ± SEM.

3.3.4 Pharmacological administration of a BAX antagonist increases neurogenesis and alters the proportion of adult-born neurons and MBP-positive putative oligodendrocytes in the ventral dentate gyrus.

Next, we used a pharmacological agent to increase adult hippocampal neurogenesis, a more translationally relevant approach. We chose to use iMac2, a synthetic small molecule designed for its activity as a BAX antagonist, ability to suppress apoptosis, as well as its low

toxicity (Bombrun et al. 2003, Peixoto et al. 2009). Mice were administered iMac2 before and during chronic CORT treatment (Figure 3.4 a). As in the previous experiment, DCX and BrdU immunohistochemistry were used to assess adult hippocampal neurogenesis (Figure 3.4 b,c). There was a trend for an increased number of DCX-positive neurons in mice treated with iMac2+CORT compared to CORT alone (27% increase, $F(1,9)=4.336$, $p=0.07$, Figure 3.4 d) and a significant increase in DCX-positive neurons with tertiary dendrites (23% increase, $F(1,9)=5.129$, $p<0.05$). However, we observed no effect on the total number of BrdU-positive neurons ($F(1,10)=1.123$, $p>0.05$, Figure 3.4 e).

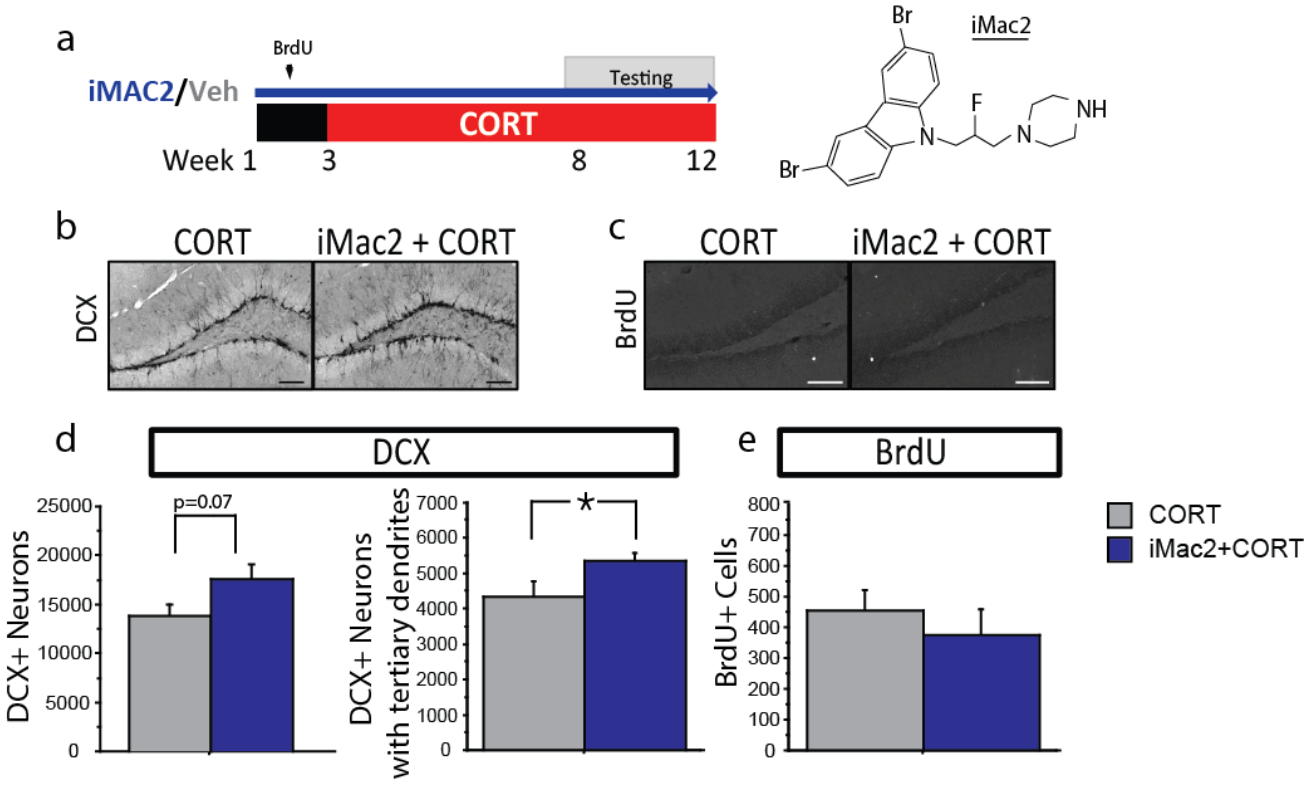

Figure 3.4 The BAX antagonist iMac2 increases adult hippocampal neurogenesis.
(a) Experimental design. (b-c) Representative images of DCX and BrdU (scale bars 100 µm). (d) There is a trend for an effect of iMac2 to increase the total number of DCX-positive neurons ($p=0.07$), and a significant effect on the number of DCX-positive neurons with tertiary dendrites (0.0498). (e) There is no difference between groups in the total number of BrdU-positive cells ($p=0.31$). n=5-6/group in all analyses. All error bars represent SEM. * $p<0.05$.

Since iMac2 was administered systemically, and could therefore act as a BAX antagonist throughout the body, we were initially concerned about effects it might have in other tissues. Whole life genetic deletion of BAX increases the weights of the spleen and thymus (Knudson et al. 1995), therefore we assessed the weights of these organs. Here, we saw no significant effect of iMac2 treatment on the weights of the spleen or thymus (all $F<4.55$, all $p>0.05$, Figure 3.5), suggesting that these organs were not largely affected.

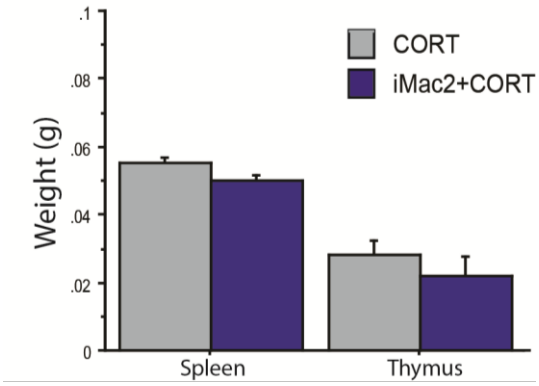

Figure 3.5 Organ **weights in mice treated with iMac2**
There is no significant effect of iMac2 treatment on the weights of the thymus (p=0.416) or spleen (p=0.086). n=3/group. Error bars represent SEM.

3.3.5 Pharmacological administration of a BAX antagonist has an anxiolytic effect in mice treated with chronic CORT.

Mice treated with CORT alone or iMac2+CORT were tested using various behavioral tests in order to determine whether iMac2 treatment affects anxiety and depression-related behavior. While there were no significant differences between CORT and iMac2+CORT groups in the open field or tail suspension tests (total distance open field $F(1,23)=0.63$, $p>0.05$; percent center distance open field $F(1,23)=0.06$, $p>0.05$; mobility tail suspension test $F(1,24)=0.809$, $p>0.05$; Figure 3.6 c), in the elevated plus maze, mice treated with iMac2+CORT displayed an

anxiolytic phenotype as compared to mice treated with CORT alone, observed by increased time spent in the open arms ($F(1,23)=9.416$, $p<0.01$) and increased open arm entries ($F(1,23)=5.802$, $p<0.05$, Figure .3.6 b).

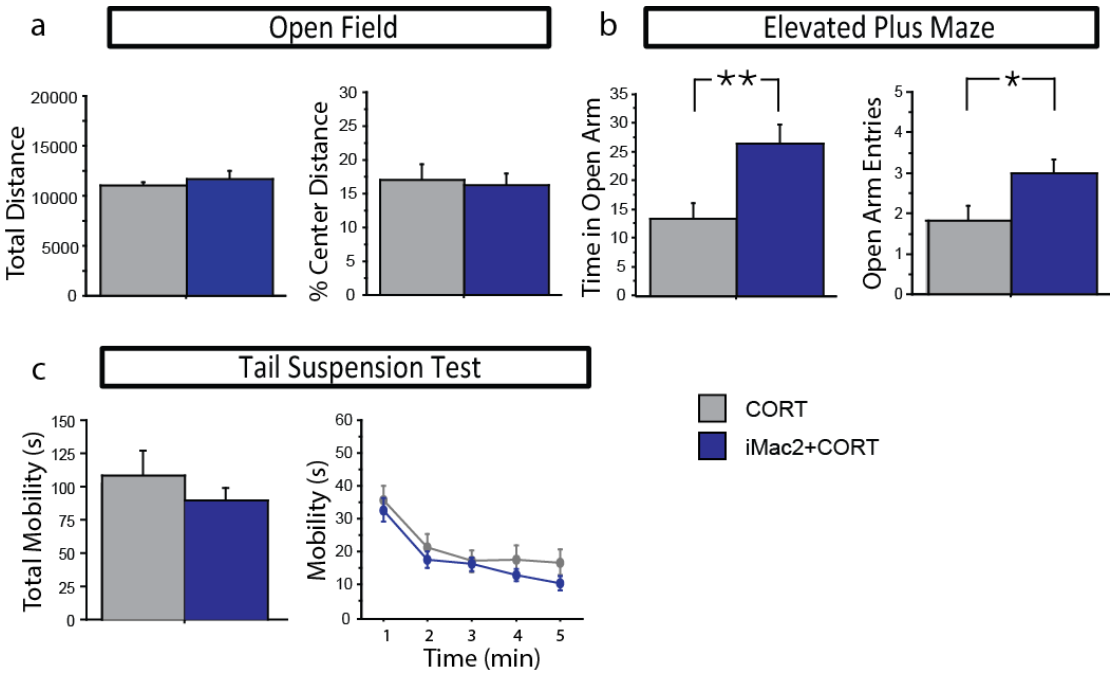

Figure 3.6 The Bax antagonist iMac2 has an anxiolytic effect in the elevated plus maze.
(a) There are no differences between groups in total distance traveled (p=0.44) or percent center distance in the open field test (p=0.81). (b) iMac2+CORT treated mice spend more time in the open arms (p=.005) and have more open arm entries (p=0.024) in the elevated plus maze compared to mice treated with CORT alone. (c) There is no difference between groups in mobility during the tail suspension test (p=0.38). In the line graph, data is represented in 1 minute bins for the duration of the test. n=12-13/group in all analyses. All error bars represent SEM. *p<0.05, ** p<0.01.

3.3.6 iMac2 does not impact behavior in a fear discrimination learning paradigm in mice treated with chronic CORT.

We were also interested in whether mice treated with iMac2 would have improved performance in a fear discrimination learning paradigm. Here, we found that both groups display different freezing levels between contexts A and B over days (CORT: $F(5,120)=13.286$

p<0.0001, iMac2+CORT: F(5,120)=21.537, p<0.0001, Figure 3.7 b). Both groups rapidly displayed differences in freezing levels between Contexts A and B, which were observed starting on Day 3 (CORT F(1,24)=4.832 p<0.05, iMac2+CORT F(1,24)=9.610, p<0.01, Figure 3.7 c). Here we observed no difference between mice treated with CORT alone or with iMac2+CORT, although the rapid learning displayed in these mice may have precluded observation of a difference between groups.

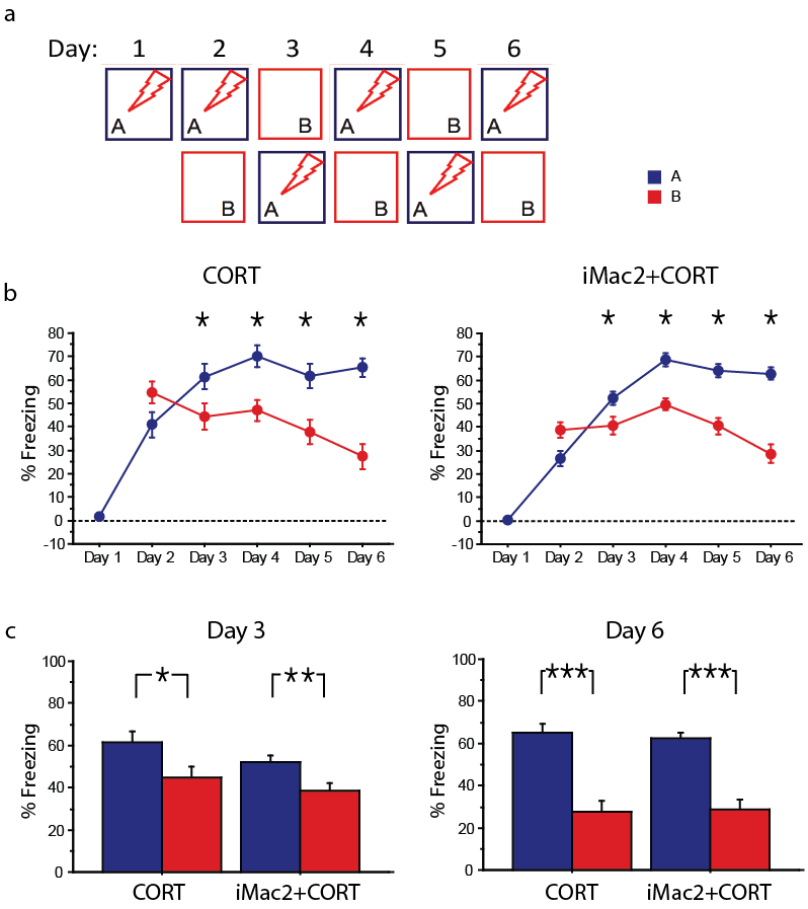

Figure 3.7 The Bax antagonist iMac2 does not affect freezing levels in a fear discrimination learning paradigm.

a) Experimental design. Context presentation was alternated on subsequent days. b) Both CORT and iMac2+CORT groups display different freezing levels between contexts A and B over days (CORT: p<0.0001, iMac2+CORT: p<0.0001) c) Both CORT and iMac2 groups display different freezing levels between contexts on Days 3 and 6 (Day 3: CORT p=0.038, iMac2+CORT p=0.0049; Day 6: CORT p<0.0001, iMac2+CORT p<0.0001). n=12-13/group. Results are presented as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001.

3.3.7 Increasing neurogenesis, either genetically or pharmacologically, alters the proportion of adult-born neurons and MBP-positive putative oligodendrocytes produced specifically in the ventral dentate gyrus.

Next, we were interested in exploring the mechanism through which increasing adult neurogenesis impacts anxiety and depression-related behavior in mice treated with chronic CORT. We were especially intrigued by the potential dissociation between the effects of increasing adult neurogenesis on cognitive, but not mood-related, behavior at baseline, and effects on mood-related, but not cognitive, behavior in mice treated with CORT. Interestingly, different regions of the hippocampus have been implicated in modulating different behaviors, with the dorsal region of the hippocampus shown to be primarily involved in cognitive related behavior, and the ventral hippocampus shown to be primarily involved in mood related behavior (Kjelstrup et al. 2002, Fanselow and Dong 2010). Furthermore, recent evidence suggests that the function of adult-born neurons in these two regions is similarly dissociated (Wu and Hen 2014). We therefore hypothesized that increasing adult hippocampus neurogenesis in mice treated with chronic CORT might specifically alter properties of the ventral hippocampus, leading to the observed effects in mood-related, but not cognitive, behaviors.

In iBax mice, we first assessed neurogenesis levels in both the dorsal and ventral hippocampus subregions using immunostaining for DCX and BrdU (Figure 3.8). Here we observed that TAM treatment had similar effects in both subregions.

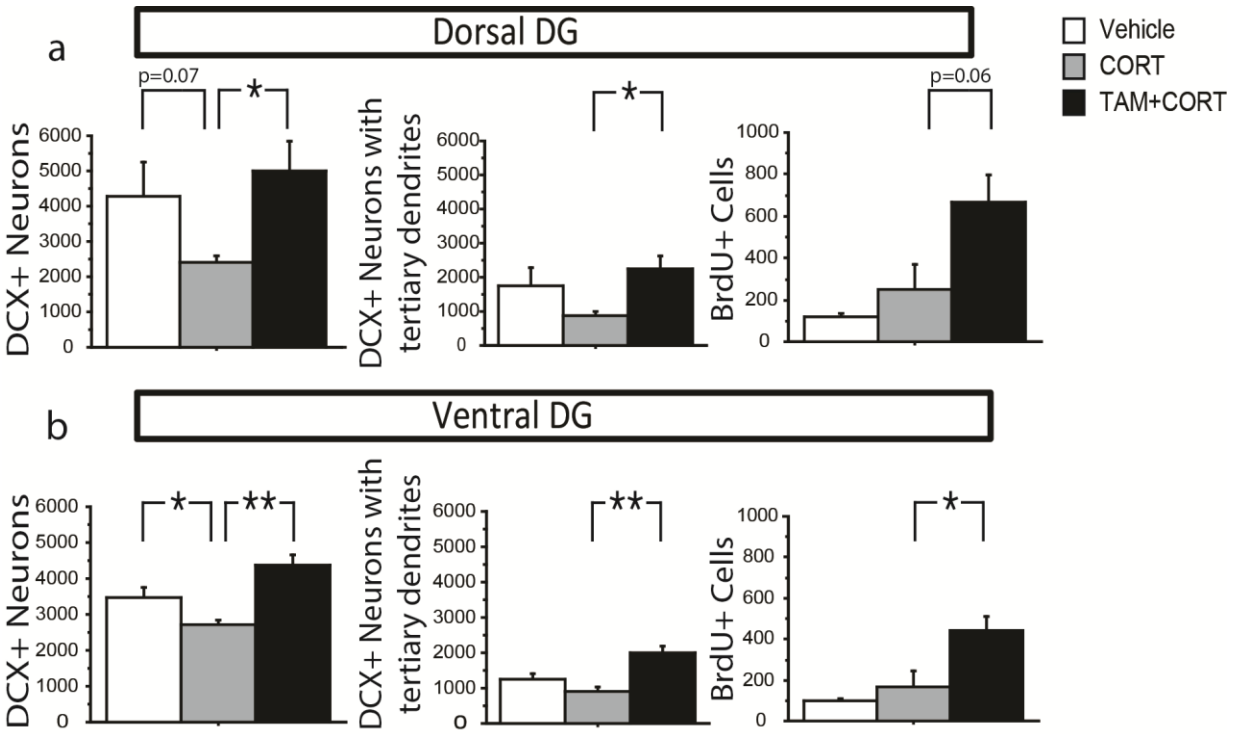

Figure 3.8 Dorsal and ventral neurogenesis data in *iBax* mice treated with CORT.
(a) Experimental design. (b,c) In the dorsal and ventral subregions of the dentate gyrus (DG), the total number of DCX-positive neurons is decreased by CORT (dorsal p=0.07, ventral p=0.039), and rescued in TAM+CORT treated mice (dorsal p=0.028, ventral p=0.002); the number of DCX-positive neurons with tertiary dendrites is increased in TAM+CORT treated mice compared to mice treated with CORT alone (dorsal p=0.013, ventral p=0.001); and the number of BrdU-positive cells is increased in TAM+CORT treated mice compared to mice treated with CORT alone (dorsal p=0.06, ventral p=0.027). All error bars represent SEM. *p<0.05, ** p<0.01.

Next, we looked more closely at the BrdU-labeled cells produced in the dorsal and ventral subregions. While neurons make up the majority of adult-born cells labeled with BrdU in the hippocampus, stem cells, astrocytes and oligodendrocytes are also found. Various environmental factors have been shown to shift the balance of adult-born cell types (van Praag et al. 2005, Dranovsky et al. 2011), including a recent finding that chronic CORT treatment increases the percent of adult-born cells labeled with BrdU that are colabeled with oligodendrocyte markers (Chetty et al. 2014). In order to determine the proportions of adult-born cell types produced in our model, we determined the identity of BrdU-labeled cells by assessing

co-expression with the neuronal marker Neuronal Nuclei (NeuN) and the oligodendrocytic marker Myelin Basic Protein (MBP) (Figure 3.9 a,b). While we saw no differences in these proportions between groups when assessing BrdU-positive cells in the dorsal dentate gyrus (all $F<0.275$, all $p>0.05$, Figure 3.9 c), we observed differences in the ventral subregion (Figure 3.9 d). Here, there appears to be a decrease in the percent of BrdU-positive cells that are colabeled with NeuN in CORT treated mice compared to vehicle, although this is not statistically significant ($F(1,6)=2.670$, $p>0.05$). However, there is a significant increase in the percent of BrdU-positive cells colabeled with NeuN in TAM+CORT treated mice (from 65±11.1% to 91±2.4%; $F(1,8)=7.987$, $p<0.05$). For the percent of adult born cells that are colabeled with MBP, we found no significant effect of CORT ($F(1,6)=2.059$, $p>0.05$), but a trend for TAM+CORT mice to have a lower percentage of BrdU-positive cells colabeled with MBP compared to mice treated with CORT alone (26±12.4% to 4±2.3%; $F(1,8)=4.633$, $p=0.06$). This data suggests that increasing adult hippocampal neurogenesis in the CORT model shifts the proportions of adult-born cells in the ventral hippocampus to relatively more neurons and fewer MBP-positive, putative oligodendrocytes.

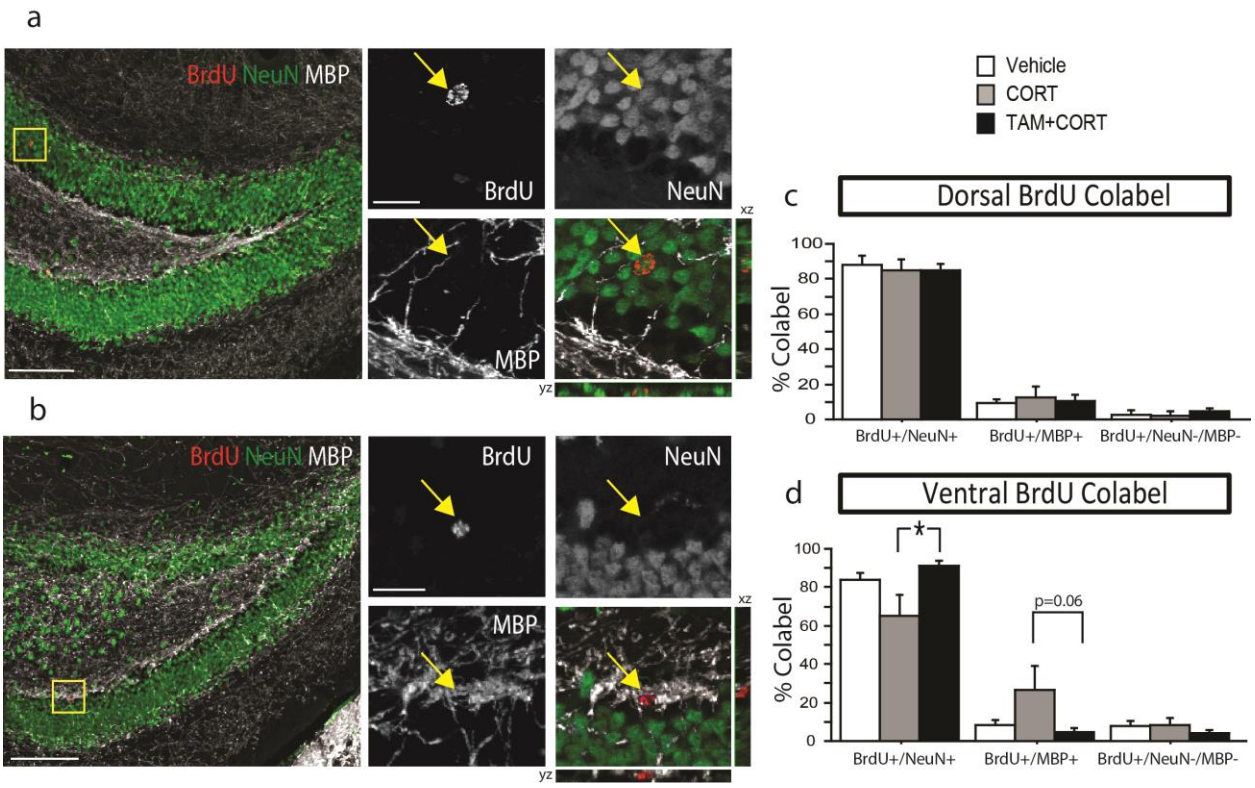

Figure 3.9 Genetic ablation of *Bax* in neural stem cells and progeny restores the balance of neuron and oligodendrocyte production in the ventral dentate gyrus of mice treated with chronic CORT.
(a,b) Representative images of BrdU colabeled with NeuN (a) and MBP (b). 20x images are on the left (scale bars 100 µm). 60x zoomed in image of area in the yellow box is on the right, including xz and yz planes in the merged image (scale bars 20 µm). (c) In the dorsal dentate gyrus, there is no difference between groups for the percent of BrdU-positive cells colabeled with NeuN, MBP or neither (all $p>0.62$). (d) In the ventral dentate gyrus, animals treated with TAM+CORT have a significantly higher percent of BrdU-positive cells colabeled with NeuN ($p=0.022$) and a trend for a lower percent of BrdU-positive cells colabeled with MBP ($p=0.06$), as compared to mice treated with CORT alone. There are no significant differences between animals treated with vehicle and CORT ($p>0.05$). n=4-7/group in all analyses. All error bars represent SEM. *$p<0.05$, ** $p<0.01$.

The same analyses were completed in mice treated with iMac2. Looking specifically in the dorsal and ventral subregions of the dentate gyrus, we observed a trend for an effect in total DCX in the dorsal but not ventral subregion, and a trend for total 3' DCX in the ventral but not dorsal subregion, but no large differences in total levels of neurogenesis between these areas (Figure 3.10).

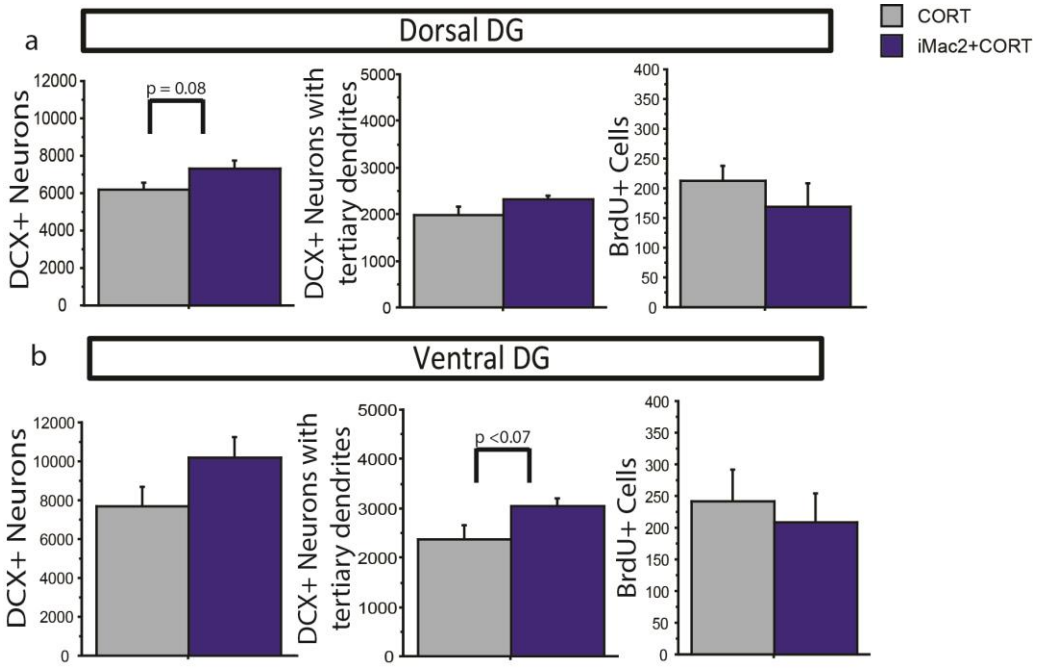

Figure 3.10 Dorsal and ventral dentate gyrus neurogenesis data in mice treated with iMac2 and CORT
(a) In the dorsal dentate gyrus, there is a trend for mice treated with iMac2+CORT to have more DCX-positive neurons than mice treated with CORT alone. (b) In the ventral dentate gyrus, there is a trend for mice treated with iMac2+CORT to have more DCX-positive neurons with tertiary dendrites than mice treated with CORT alone. n=5-6/group in all analyses. All error bars represent SEM.

Next, we performed colabeling of BrdU with NeuN and MBP (Figure 3.11 a,b). Just as in *iBax* mice, we found no differences in the dorsal dentate gyrus (all $F<0.637$, all $p>0.05$, Figure 3.11 c), however in the ventral dentate gyrus, iMac2 treatment increased the percent of BrdU-positive cells colabeled with NeuN (from 61±3.7% to 76±4.4%, $F(1,10)=7.189$, $p<0.05$), and decreased the percent colabeled with MBP (from 27±2.1% to 13±3.2%, $F(1,10)=13.095$, $p<0.01$, Figure 3.11 d). Here, even though there is no change in the total number of BrdU-positive cells, iMac2 treatment shifts the balance of neurons and oligodendrocytes produced.

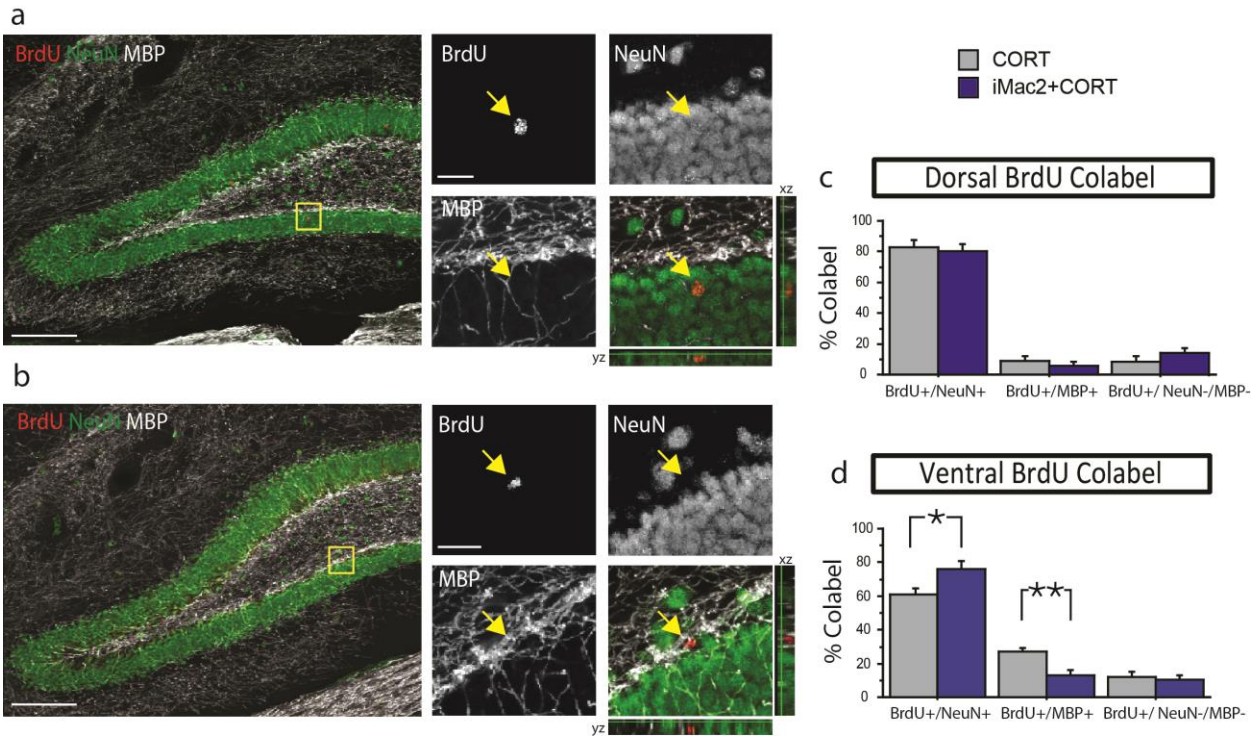

Figure 3.11 iMac2 shifts the balance of neurons and oligodendrocytes produced in the ventral dentate gyrus. (a-b) Representative images of BrdU colabeled with NeuN (a) and MBP (b). 20x images are on the left (scale bars 100 µm). 60x zoomed in image of area in the yellow box is on the right, including xz and yz planes in the merged image (scale bars 20 µm). (c) In the dorsal dentate gyrus, there are no differences in the percent of BrdU-positive cells colabeled with either NeuN ($p=0.73$) or MBP ($p=0.44$). (d) In the ventral dentate gyrus, iMac2+CORT treated mice have a significantly higher percentage of BrdU-positive cells colabeled with NeuN ($p=0.023$) and lower percentage of BrdU-positive cells colabeled with MBP ($p=0.005$). n=5-6/group in all analyses. All error bars represent SEM. *$p<0.05$, ** $p<0.01$.

Together, these data from both iBax mice, and mice treated with iMac2, suggest that increasing neurogenesis in mice treated with CORT specifically alters the balance of cell types produced in the ventral, but not dorsal, hippocampus. Since the ventral hippocampus is also the region of the hippocampus involved in mood-related behavior, this provides a possible mechanism through which increasing neurogenesis in mice treated with CORT might impact behavior.

3.4 Discussion

Here we have shown that increasing adult hippocampal neurogenesis is sufficient to provide resilience to chronic CORT administration. In *iBax* mice, we genetically blocked cell death in adult-born cells from neural precursors, which increases the number of adult-born neurons that reach maturation. In this way, we specifically mimicked this single property of antidepressants to increase adult neurogenesis, and showed that it is sufficient for antidepressant-like behavioral effects in mice treated with chronic CORT, although interestingly, we do not see an effect of increasing neurogenesis on performance in a fear discrimination learning task. We suggest the possibility that increasing adult hippocampal neurogenesis may impact behavior by shifting the balance of neuronal and oligodendrocyte production, which we observe specifically in the ventral dentate gyrus. Additionally, we support these findings with similar results using a pharmacological BAX inhibitor, iMac2, showing that chronic administration of this drug also decreases anxiety, increases adult neurogenesis and shifts the balance of neuronal and oligodendrocyte production specifically in the ventral dentate gyrus.

3.4.1 Increasing adult hippocampal neurogenesis prevents the effects of chronic CORT on anxiety and depression-related behavior, but not HPA axis regulation.

In *iBax* mice, we show that chronic CORT has anxiogenic and depressive effects in a subset of behavioral tests, which are prevented in mice with increased adult hippocampal neurogenesis (Figure 3.2). Conversely, in tests in which CORT has no effect, increasing neurogenesis also has no effect. In this case, the behavior of all groups is similar to controls, and mimics the lack of an effect under baseline, no-stress conditions (Chapter 2). The disparity between the effects under these two conditions, baseline and chronic CORT, suggests that

increased neurogenesis only affects anxiety and depression-related behavior when animals are in a stressed-like state.

This disparity parallels the effects of antidepressants when given to certain strains of mice where antidepressants do not affect behavior at baseline, but do following chronic CORT or chronic unpredictable mild stress (David et al. 2009, Surget et al. 2009). Furthermore, in humans, antidepressants have been shown to be more effective in severely depressed patients (Fournier et al. 2010), suggesting a similar disparity. Since adult hippocampal neurogenesis is a component of the mechanism of action of antidepressants, the constraint that antidepressants only affect behavior in depressed patients might also apply to increasing adult hippocampal neurogenesis, such that this manipulation only affects 'stressed' mice.

One prevailing hypothesis in the literature is that adult neurogenesis affects anxiety and depression-related behaviors through altered regulation of the HPA axis, since the absence of adult neurogenesis impairs HPA axis regulation following acute stress (Schloesser et al. 2009, Snyder et al. 2011) and prevents the effects of antidepressants on the HPA axis (Surget et al. 2011). While these studies show that intact adult hippocampal neurogenesis is required for proper HPA axis activity, our data suggests that increasing adult hippocampal neurogenesis is not sufficient to affect this process (Figure 3.2). This data therefore suggests that increased neurogenesis likely can affect behavior through a mechanism independent of HPA axis regulation. Nevertheless, we do note that under different stress conditions, where the endogenous HPA axis is active, increased neurogenesis could affect behavior through an additional mechanism involving the HPA axis.

3.4.2 Increasing adult hippocampal neurogenesis in mice treated with chronic CORT does not impact fear discrimination learning behavior.

Interestingly, in mice treated with chronic CORT we do not see an effect of increasing adult hippocampal neurogenesis on performance in fear discrimination learning paradigms in either *iBax* mice (Figure 3.3) or mice administered iMac2 (Figure 3.7). In the *iBax* experiment, no groups showed different freezing levels in the two contexts that reached statistical significance, while in the iMac2 experiment, all groups rapidly showed significantly different freezing levels. Different behavior between these two strains of mice (the mixed strain *iBax* mice and pure C57BL/6 mice treated with iMac2) is not entirely unexpected since different strains of mice have been shown to behave differently in memory-related behavioral tests (Crawley et al. 1997). The lack of an effect of increasing adult hippocampal neurogenesis on behavior in these experiments may be because the tasks were not optimized to detect differences between these groups, since all mice appear to either have not learned (in the *iBax* experiment) or to have learned very quickly (in the iMac2 experiment). Alternatively, neurogenesis may have different effects on the circuitry underlying pattern separation at baseline or in mice treated with chronic CORT, a possibility that will be further discussed in Chapter 4.

3.4.3 Increasing adult hippocampal neurogenesis shifts the proportions of adult-born neurons and oligodendrocytes produced.

Different environmental factors have been shown to shift the balance of adult-born cell types (van Praag et al. 2005, Wong and Herbert 2006, Dranovsky et al. 2011), and a particularly interesting recent study showed that chronic CORT treatment decreases the percent of adult-born

cells that become neurons, while increasing the percent that become oligodendrocytes (Chetty et al. 2014).

Adult-born oligodendrocytes are produced by NG2-positive oligodendrocyte precursor cells, which are located throughout the brain, including in the dentate gyrus (Kang et al. 2010). It is unclear whether oligodendrocytes can also be produced from the Nestin-positive stem cells in the SGZ that give rise to adult-born granule cells. This was observed in one study using a Nestin-CreERT2 transgenic mouse line crossed to a reporter line to label adult-born cells from the Nestin lineage, some of which were found to express MBP in adult mice treated with CORT (Chetty et al. 2014). However, another study characterizing the cell types produced by Nestin-positive stem cells in the adult SGZ did not detect any oligodendrocytes, although this was done in naïve mice, not treated with chronic CORT (Bonaguidi et al. 2011). This discrepancy may be because production of oligodendrocytes in no-stress conditions is very low and therefore was not detected in the second study, or because these two studies used different Nestin-CreERT2 transgenic mouse lines, which have been shown to label Nestin-positive stem cells with different levels of efficiency and ectopic expression (Sun et al. 2014). Whether oligodendrocytes are produced from Nestin progenitor cells is therefore unclear.

In the experiments presented here, we did not observe significant effects of CORT on the proportions of adult-born neurons and oligodendrocytes produced, although this may be because BrdU was injected weeks before the onset of CORT treatment. Nevertheless, we found that genetically or pharmacologically increasing adult hippocampal neurogenesis modulated the proportions of neurogenesis and oligodendrogenesis in the opposite directions than expected due to CORT, and also reversed the behavioral effects of CORT. Notably, irregular myelination has been detected in several mental illnesses (reviewed in (Edgar and Sibille 2012)), therefore

maintaining appropriate levels of myelination by balancing neuronal and oligodendrocyte production may be important for mood regulation.

The role of oligodendrogenesis in the adult dentate gyrus, and how this process may impact behavior, is not fully understood. Although dentate granule cells themselves are not myelinated (Blackstad and Kjaerheim 1961), axons that project onto granule cells from the entorhinal cortex (Jensen et al. 1999) as well as mossy cells (Ribak et al. 1985) are myelinated. Whether oligodendrogenesis in the adult hippocampus is involved in incorporation of adult-born neurons into existing circuits is unknown, but provides an intriguing hypothesis for the importance of maintaining balance in the production of neurons and oligodendrocytes. Our results extend the hypothesis that changes in myelination may underlie the behavioral effects of stress that lead to psychiatric illness (Edgar and Sibille 2012, Chetty et al. 2014), and further suggest that manipulations to prevent the effects of stress on oligodendrogenesis may provide resilience.

Interestingly, we observed high levels of myelin basic protein (MBP) staining in the SGZ, an area with few myelinated axons. In fact, the hilus has been previously identified as a hippocampal subregion with a high density of oligodendrocytes, despite relatively few myelinated axons (Vinet et al. 2010). There are a few possible reasons that oligodendrocytes would be located in the SGZ. First, the SGZ is a niche for the production of adult-born granule cells from Nestin-positive progenitors. Oligodendrocyte production from oligodendrocyte precursors or nestin-positive progenitors may thrive on similar factors as neurogenesis, which are present in the SGZ. For example, oligodendrocytes are often found adjacent to blood vessels (Vinet et al. 2010), which are more dense in the SGZ than elsewhere in the hippocampus. Second, increasing evidence shows that oligodendrocytes have receptors and respond to various

neurotransmitters (Karadottir et al. 2005), and it has therefore been suggested that oligodendrocytes with cell bodies in the hilus may receive input that could affect processes that myelinate entorhinal axons in the molecular layer of the dentate gyrus (Vinet et al. 2010).

It is particularly interesting that differences in the proportion of adult-born neurons and oligodendrocytes produced are observed specifically in the ventral, but not dorsal dentate gyrus. Numerous studies have now shown that the dorsal and ventral hippocampus have different genetic expression, connectivity and function (Fanselow and Dong 2010, Kheirbek et al. 2013). Furthermore, many studies have observed larger effects of chronic stressors and antidepressants on ventral than dorsal neurogenesis (Tanti and Belzung 2013, Wu and Hen 2014, Wu et al. 2014). Although we do not see a large difference due to the effects of chronic CORT on total levels of neurogenesis in the dorsal and ventral subregions (Figures 3.8 and 3.10), we do see an effect of increasing neurogenesis on the balance of adult-born neuron and putative oligodendrocyte production specifically in the ventral dentate gyrus (Figures 3.9 and 3.11), supporting the hypothesis that neurogenesis in the ventral subregion is particularly important for anxiety and depression-related behavior.

3.4.4 iMac2 is a candidate anxiolytic that acts by increasing adult hippocampal neurogenesis.

iMac2 is a small molecule in the class of 2,6-dibromocarbazole piperazine derivatives of 2-propanol, which act as BAX antagonists (Bombrun et al. 2003). A similar molecule in this class has been previously shown to increase adult hippocampal neurogenesis (Serono compound 1 (Pieper et al. 2010)), and we have confirmed that iMac2 increases adult hippocampal neurogenesis in C57BL/6 mice (unpublished results). While genetic deletion of *Bax* is sufficient

to block cell death in adult-born neurons, many other cell types require deletion of both *Bax* and *Bak*, another pro-apoptotic gene, in order to block cell death (Takeuchi et al. 2005, Biswas et al. 2010). In fact, the only other tissues with increased weights following whole life genetic deletion of *Bax* are the spleen and thymus (Knudson et al. 1995). Here we observed no change in the weights of these organs following systemic iMac2 administration (Figure 3.5), suggesting that the previously observed effects may be due to the absence of *Bax* during development, or that iMac2 does not infiltrate these tissues. These results and the literature suggest the possibility that systemic administration of a BAX antagonist such as iMac2 may selectively impact neurogenesis in the adult, however more extensive testing is needed to support this claim.

Genetic deletion of *bax* has a larger effect on neurogenesis than iMac2 treatment, however even the smaller effect in the iMac2 experiment is sufficient to impact behavior. In the iMac2 experiment, we see an increase in neurogenesis as assessed by DCX, but not by BrdU. This discrepancy suggests to us that the cellular populations represented by these two measures may have been impacted differently by iMac2. BrdU was injected after 1 week of iMac2 treatment, and the majority of BrdU-positive cells were likely born around the time of BrdU injection. On the other hand, the population of DCX-positive cells assessed was born 1-3 weeks before sacrifice. It is therefore possible that the effects of iMac2 on neurogenesis increased throughout treatment, leading to a larger effect on the DCX population.

Additional understanding of the developmental stage at which adult-born neurons contribute to mood-related behavior will also be useful. Here, levels of neurogenesis were increased 7-9 weeks before behavioral testing (7 weeks in the iMac2 experiment and 9 weeks in the *iBax* experiment). At the time of behavioral testing, there were likely increased levels of adult-born neurons aged 0-7 or 0-9 weeks. Development of more sophisticated techniques could

allow increase of adult-born neurons only within a specific time window, allowing determination of whether there is a precise age range at which adult-born neurons contribute to mood-related behavior. Precise ablation studies probing the contribution of adult-born cells to cognitive tasks have identified 4 to 6 weeks as the age range during which maturing neurons influence these tasks (Kee et al. 2007, Denny et al. 2012), but it is not yet known whether adult-born cells in the same age-range contribute to mood-related behavior.

While the results presented here suggest that increasing adult hippocampal neurogenesis can protect against the effects of a chronic stressor, it remains to be seen whether this manipulation can rescue behavior if neurogenesis is increased *after* a chronic stressor. As antidepressants are generally prescribed after the onset of depression, this is an important point to be addressed for the translational feasibility of targeting neurogenesis. Preliminary data addressing this point is presented in Appendix A, and suggest that increasing adult hippocampal neurogenesis may be sufficient to rescue the anxiety phenotype of chronic CORT in the elevated plus maze. Additionally, imaging tools being developed to detect levels of adult hippocampal neurogenesis in depressed patients may allow increased adult hippocampal neurogenesis to serve as a biomarker for antidepressant efficacy (Couillard-Despres and Aigner 2011, Tanti and Belzung 2013).

The data presented here suggest that neural stem cells in the adult mammalian brain provide a resource that may be harnessed to treat various disorders. Expanding the production of adult-born neurons may be beneficial for the treatment of depression and anxiety disorders.

3.5 Involvement

I was directly involved in all experiments presented in this chapter, with input from many members of the Hen lab and assistance from several lab volunteers.

Chapter 4: Discussion

4.1 Summary of results

This thesis describes the effects of increasing adult neurogenesis in mice under three conditions: baseline, voluntary exercise and chronic CORT. Interestingly, increasing neurogenesis has different behavioral effects in each of these conditions: improving performance in contextual fear discrimination tasks at baseline, increasing exploratory behavior in mice exposed to voluntary exercise, and affecting anxiety and depression-related behavior in mice treated with chronic CORT. In these experiments, two manipulations were used to increase adult hippocampal neurogenesis: a genetic method using *iBax* mice to specifically increase survival of adult neuronal stem cells, and iMac2 a systemically administered BAX antagonist. In mice treated with chronic CORT, iMac2 replicated a subset of the behavioral effects observed in *iBax* mice, providing an anxiolytic effect in the elevated plus maze. Furthermore, increasing adult hippocampal neurogenesis, either genetically or pharmacologically, in mice treated with chronic CORT altered the proportions of neurons and oligodendrocytes produced in the ventral hippocampus, providing a potential mechanism through which increasing adult hippocampal neurogenesis impacts behavior, and suggesting regional specificity of the effects of this manipulation in mice treated with chronic CORT.

4.2 Roles of adult hippocampal neurogenesis under different environmental conditions

It was found that increasing adult hippocampal neurogenesis has different behavioral effects under different environmental conditions: effects on mood-related behavior in mice

treated with CORT, effects on pattern separation in mice at baseline, and effects on exploration in mice exposed to voluntary exercise. At first glance, it appears that these different effects could be because the initial and altered levels of neurogenesis are at different absolute values for each condition. Neurogenesis levels are relatively low in mice treated with CORT, and high in mice exposed to voluntary exercise, as compared to mice at baseline. Together, a possible framework could be proposed: changes in neurogenesis at low levels (by increasing neurogenesis in mice treated with CORT) would affect mood-related behavior, changes in neurogenesis at intermediate levels (by increasing neurogenesis at baseline) would affect pattern separation, and changes in neurogenesis at high levels (by increasing neurogenesis in mice exposed to voluntary exercise) would affect exploration. However, experimental data from mice at baseline with ablated neurogenesis argues against this explanation; the absolute values of neurogenesis levels in these groups fall closest to the low levels of neurogenesis in the above framework, yet these experiments have found differences in pattern separation (Figure 2.5), but generally not in mood-related behavior (at least not without additional interventions) (Santarelli et al. 2003). While additional experiments will be informative to further explore this proposed framework, it is not entirely consistent with the published literature.

Instead, the different observed behavioral effects may be because the environmental conditions used are known to have various different physiological effects, which may alter the integration of adult-born neurons into hippocampal circuits. Physiological effects of each condition provide potential mechanisms through which increasing adult hippocampal neurogenesis may affect different behaviors depending on environmental condition.

4.2.1 Pattern separation is improved in animals with increased adult hippocampal neurogenesis at baseline, but not in mice treated with chronic CORT.

At baseline, increasing adult hippocampal neurogenesis enhances ACSF-LTP (Figure 2.4) and improves performance in contextual discrimination learning paradigms (Figures 2.7 and 2.8), but does not impact exploratory, anxiety or mood-related behavior (Figure 2.10).

As presented in Chapter 1, adult-born neurons have been hypothesized to contribute to pattern separation in two ways. First, in a cell autonomous manner, the increased excitability of adult born cells may allow them to encode information in a unique way (Aimone et al. 2010). Here, *iBax* mice with increased adult hippocampal neurogenesis display enhanced ACSF-LTP, as well as enhanced performance in the discrimination learning paradigm, further supporting this hypothesis.

Second, the dentate gyrus is thought to be involved in pattern separation partially through its sparse activity (O'Reilly and McClelland 1994). Adult neurogenesis has been hypothesized to contribute to the sparse activity of the dentate gyrus through the addition of young, excitable cells that synapse onto inhibitory interneurons throughout the dentate gyrus, increasing inhibitory tone (Kheirbek et al. 2012, Lacefield et al. 2012, Song et al. 2012). Since voltage-sensitive dye imaging in *iBax* mice has shown that increasing adult hippocampal neurogenesis decreases activity in the dentate gyrus (Ikrar et al. 2013), increasing adult hippocampal neurogenesis may therefore improve pattern separation by increasing sparseness in the dentate gyrus.

It is interesting that behavioral effects are observed in the pattern separation task, which has been shown to be dependent on neurogenesis in the dorsal hippocampus, but behavioral effects are not seen in mood-related tasks, which have been shown to involve neurogenesis in the ventral hippocampus (Wu and Hen 2014). More precise analysis of ACSF-LTP and hippocampal

activity levels along the longitudinal axis will shed light on whether hippocampal circuitry is differentially affected in the dorsal and ventral subregions. Potential downstream brain regions that receive projections from the dorsal hippocampus and may mediate the effects of increased levels of adult hippocampal neurogenesis on fear discrimination learning behavior are discussed in Section 4.3.

4.2.2 Exploratory behavior is increased in mice exposed to voluntary exercise, while it is not impacted in mice at baseline or exposed to chronic CORT.

Various environmental factors increase adult hippocampal neurogenesis, including exposure to learning tasks, antidepressants and voluntary exercise. Importantly, these environmental factors induce many changes in the hippocampus, which may affect the function and behavioral contribution of adult-born neurons alongside increases in adult-born cell number.

Voluntary exercise has been shown to enhance short and long term potentiation in the dentate gyrus, change expression levels of various receptors, and increase dendritic complexity and spine density of dentate granule cells (Fordyce and Farrar 1991, van Praag et al. 1999, Farmer et al. 2004, Eadie et al. 2005). Due to these effects of voluntary exercise, combining an increase in adult-born cell number with voluntary exercise might change the functional contribution of the increased number of adult-born cells. Under these conditions, it is likely that increasing adult hippocampal neurogenesis could impact the hippocampal network in a manner that is different from the impact at baseline, or in mice treated with chronic CORT. In future experiments, ACSF-LTP, voltage sensitive dye imaging, or calcium imaging of the dentate gyrus could be used to test this point, in order to determine whether there is a different magnitude effect in mice exposed to voluntary exercise.

Increasing adult hippocampal neurogenesis in mice that have experienced voluntary exercise increases exploration in the open field test (Figure 2.13), but has no effects on anxiety or depression-related behaviors in other tests (Figure 2.14). Mice that experienced voluntary exercise were not tested in the discrimination learning paradigm. Since mice that have experienced voluntary exercise display enhanced performance in various memory related tasks (Fordyce and Farrar 1991, van Praag et al. 1999, van Praag et al. 2005), a more difficult discrimination learning task would be necessary to test the role of increasing adult hippocampal neurogenesis in these mice.

It is unknown whether the effect on exploration is unique to voluntary exercise, or could be achieved with other conditions that increase granule cell plasticity, such as other forms of environmental enrichment or increased levels of trophic factors, like BDNF. Additional experiments using these manipulations would provide insight into how these different manipulations affect integration of adult born neurons into the hippocampal circuitry to affect different behavioral constructs.

4.2.3 Anxiety and depression-related behavior are impacted by increased adult hippocampal neurogenesis in mice treated with chronic CORT, but not at baseline or in mice exposed to voluntary exercise.

In Chapter 3, data was presented showing that in mice treated with chronic CORT, increasing adult hippocampal neurogenesis does not impact behavior in a discrimination learning paradigm (Figures 3.3 and 3.7) , but is sufficient to prevent the effects of CORT on anxiety and depression-related behavior (Figure 3.2 and 3.6). The different effects of increasing adult hippocampal neurogenesis in mice treated with chronic CORT compared to mice at baseline or

exposed to voluntary exercise suggests that in the chronic CORT condition, adult born neurons contribute to hippocampal function in an additional, unique way. The lack of an effect at baseline also parallels the lack of an effect of antidepressants on mood in healthy humans, suggesting that a similar constraint may prevent baseline effects of these manipulations.

The effects of increasing adult hippocampal neurogenesis on anxiety and depression-related behavior were seen specifically to prevent the effects of CORT. Chronic CORT treatment, like other chronic stressors, leads to many changes in the hippocampus, including decreased hippocampal CREB phosphorylation, BDNF levels and dendritic complexity in CA3 pyramidal cells (Woolley et al. 1990, Gourley et al. 2008, Gourley and Taylor 2009). Reduced dendritic complexity of CA3 pyramidal cells due to other stressors has been shown to be dependent on excitatory neurotransmission (McEwen and Magarinos 1997, McEwen 1999, Christian et al. 2011). Since increasing adult hippocampal neurogenesis reduces excitability (Ikrar et al. 2013), this might be sufficient to prevent the effects of CORT on dendritic complexity, providing a candidate mechanism through which increasing adult hippocampal neurogenesis might prevent the effects of chronic CORT.

There is also evidence that CORT directly affects adult-born cells. Knockout of the glucocorticoid receptor (GR) specifically in adult born cells accelerates maturation and migration, and increases dendritic spines, mossy fiber boutons, and basal excitability (Fitzsimons et al. 2013). These physiological changes observed following deletion of GR are candidate properties that may be affected by chronic CORT treatment. Any of these effects of CORT on hippocampal circuit processing may be reversed by increasing adult hippocampal neurogenesis.

Since involvement of the hippocampus and specifically of adult hippocampal neurogenesis in cognitive and mood related tasks has been separated along the longitudinal axis

(Fanselow and Dong 2010, Wu and Hen 2014), increasing adult hippocampal neurogenesis in mice treated with chronic CORT might specifically impact hippocampal function in the ventral region, which is involved in anxiety and depression-related behavior, while not affecting hippocampal function in the dorsal region, which is involved in cognitive related behavior.

Interestingly, in mice treated with chronic CORT, a difference in the effects of increasing adult hippocampal neurogenesis was observed between the dorsal and ventral hippocampal regions. Increasing adult hippocampal neurogenesis affects the proportion of neurons and oligodendrocytes produced in the ventral hippocampus, but not in the dorsal hippocampus, suggesting that this manipulation might specifically affect this portion of the hippocampus. A recent study has shown that stress or CORT treatment shifts this balance in the opposite direction (Chetty et al. 2014). Although we did not observe significant effects of CORT treatment in the *iBax* experiment (Figure 3.9), we note that in this experiment, BrdU was injected five weeks before the onset of CORT administration, which may have prevented observation of an effect. A reversal of the expected effect of CORT on oligodendrogenesis could represent a mechanism through which increasing adult hippocampal neurogenesis provides resilience in this stress model, producing effects on anxiety and depression-related behavioral tests in this condition, but not in mice at baseline or exposed to voluntary exercise. In these other conditions, increasing adult hippocampal neurogenesis had no effect on the proportion of adult-born neurons produced (Figures 2.2 h and 2.12 c), suggesting that this effect is specifically found in mice treated with chronic CORT.

A precise understanding of how CORT shifts the production of adult-born cells from neurons towards oligodendrocytes is not known. Both oligodendrocyte and neuronal precursors have been shown to express GR (Garcia et al. 2004, Matsusue et al. 2014), suggesting that

proliferation could be directly impacted by CORT. Although the effects of CORT on proliferation in these two cell types is in opposite directions, GR has been shown to impact various genes in opposite direction in different tissues, depending in part on other transcription factors present (Beato et al. 1995, Luca et al. 2013).

While causation has not been determined, our work adds to the hypothesis that changes in neurogenesis and oligodendrogenesis in the adult hippocampus might impact anxiety and depression-related behavior. Along with providing myelination, oligodendrocytes are involved in transport along and survival of axons, possibly by providing trophic support (Tachikawa et al. 2004, Nave 2010). Mounting evidence shows that oligodendrocytes are important for proper axonal function in a more intricate way than previously thought. Various stressors have been shown to affect levels of myelination. While social isolation has been shown to decrease myelin in some brain regions (Liu et al. 2012), stress during pregnancy has been shown to increase myelination in pups during development (Wiggins and Gottesfeld 1986). Notably, irregular myelination in humans has been detected in several mental illnesses (reviewed in (Edgar and Sibille 2012)), therefore a precise level of myelination may be important for proper brain function.

There are two possible ways that increasing adult hippocampal neurogenesis could prevent detrimental effects of increased oligodendrogenesis. First, as discussed in Chapter 3, it may be that the proportions of neurons and oligodendrocytes produced in the adult hippocampus need to be at a fixed ratio for proper function. For example, adult-born oligodendrocytes could be involved in the integration of adult born neurons into hippocampal circuitry in such a way that requires balance. Second, levels of adult hippocampal neurogenesis may affect oligodendrocytes via changes in hippocampal activity. A relevant recent study has shown that increasing neuronal

activity can increase myelination (Gibson et al. 2014). Since increasing adult neurogenesis has been shown to decrease activity in the hippocampus (Ikrar et al. 2013), increasing adult neurogenesis may decrease myelination through changes in overall activity levels of the dentate gyrus. Additional experiments to assess the effects of CORT and increased levels of neurogenesis on myelination in the dentate gyrus may provide evidence for one of these possibilities. Further work is needed to determine how shifts in oligodendrogenesis and myelination affect behavior, but this is an interesting emerging concept that may be related to the behavioral effects observed due to increasing adult hippocampal neurogenesis in mice treated with chronic CORT.

4.2.4 Overview of effects of increasing neurogenesis: comparison of pattern separation and mood-related behavior.

As discussed above, different behavioral effects of increasing adult hippocampal neurogenesis are observed under three conditions (baseline, voluntary exercise, and chronic CORT), suggesting different underlying mechanisms. However, it has been suggested that deficits or improvements in pattern separation might affect anxiety and depression-related behavior, raising the possibility that the observed behavioral effects may stem from similar changes in hippocampal function.

A role for adult hippocampal neurogenesis in the pathophysiology of anxiety disorders, such as post-traumatic stress disorder (PTSD), has been hypothesized based on the possibility that stress-induced decreases in neurogenesis may impair pattern separation, making an afflicted individual more likely to associate every day experiences with memories from a traumatic event (Kheirbek et al. 2012). Furnishing this hypothesis, patients with panic disorder display stronger

conditioned generalization as assessed by startle electromyography (EMG) in a fear-generalization paradigm (Lissek et al. 2010). Conversely, it is therefore possible that the observed effect of increasing adult hippocampal neurogenesis on anxiety and mood-related behavior in mice treated with chronic CORT may be due to improved pattern separation, even though performance in the contextual fear discrimination learning paradigm is not affected. Alternatively, we cannot rule out the possibility that changes in mood could affect pattern separation, even though we have not seen any evidence for this hypothesis. Nevertheless, these possibilities suggest that increasing adult hippocampal neurogenesis in mice exposed to different conditions may affect hippocampal processing in similar ways, but may lead to different behavioral effects via distinct downstream circuits.

4.3 Possible downstream circuits through which adult hippocampal neurogenesis may affect cognitive, exploratory and mood-related behavior

The dorsal and ventral regions of the hippocampus, which primarily modulate cognition and mood respectively, project to distinct brain regions, suggesting that distinct downstream circuits mediate the effects of dorsal and ventral hippocampal manipulations. Since increasing adult hippocampal neurogenesis affects either cognitive or mood-related behavior under different conditions, these effects are likely mediated by different downstream circuits.

4.3.1 Candidate downstream circuits through which adult hippocampal neurogenesis may affect memory-related behavior.

The dorsal hippocampus is thought to contribute to cognitive and memory related-tasks, since dorsal hippocampal lesions impair performance in memory-related tasks (Moser et al.

1995, Pothuizen et al. 2004). Furthermore, ablation of adult-born neurons specifically in the dorsal dentate gyrus impairs performance in contextual fear discrimination learning (Wu and Hen 2014). The dorsal hippocampus sends projections to the lateral septum, thalamus, mammillary complex, retrosplenial cortex, and anterior cingulate cortex, in addition to the perirhinal cortex and lateral entorhinal cortex (Wyss and Van Groen 1992, Namura et al. 1994, Witter 2006).

One primary subcortical candidate for mediating hippocampal influence on memory is the retrosplenial cortex, which has been shown to be involved in spatial (Vann and Aggleton 2002) and contextual (Keene and Bucci 2008) memory tasks. Furthermore, intermediate early gene expression in the retrosplenial cortex is increased following learning tasks, an effect which is blocked by hippocampal inactivation, implying a role for hippocampal input to this region (Kubik et al. 2012). Another projection that may be involved in memory is from the hippocampus to the anterior cingulate (Fanselow and Dong 2010), since this region has been shown to be involved in storage of memory associated with contextual fear conditioning (Frankland et al. 2004). Other dorsal hippocampal projections may be involved in exploration (as discussed in Section 4.3.2). This involvement in exploration may be critically linked to the ability to form a cognitive map of an environment, which may be critical for memory (Muller et al. 1996), and therefore may also contribute to behavior in cognitive tasks.

4.3.2 Candidate downstream circuits through which adult hippocampal neurogenesis may affect exploratory behavior.

The portion of the hippocampus that impacts exploratory behavior is not well defined. Increased exploration in the open field has been observed following lesion of the hippocampus

(Jarrard and Bunnell 1968), although this effect is not always observed (Markowska and Lukaszewska 1981). In one set of studies, it was shown that while lesions of the dorsal or ventral hippocampus increase exploration (Rossi-Arnaud and Ammassari-Teule 1992), a larger portion of the ventral hippocampus than the dorsal hippocampus must be lesioned to affect locomotion (Ammassari-Teule and Passino 1997). Further supporting the importance of the dorsal hippocampus in modulating exploratory behavior, in a recent paper from our lab, optogenetic stimulation specifically of the dorsal dentate gyrus increased exploratory behavior in the open field (Kheirbek et al. 2013). This manipulation is also blocked by a dopamine receptor 1 antagonist (Kheirbek et al. 2013), suggesting a possible downstream mechanism through dopaminergic cells.

A primary downstream candidate brain region for hippocampal effects on exploratory behavior is the ventral tegmental area (VTA), which contains dopaminergic cells, and receives input from the hippocampus through a relay in the lateral septum (Swanson and Kalivas 2000, Luo et al. 2011). As presented in Appendix B, preliminary results of optogenetic stimulation of hippocampal projections to the lateral septum leads to increased exploration in the open field (Figure B.2). However, following testing in the open field, stimulated mice display high levels of cFos in the dentate gyrus (Figure B.3), which may lead to activation of additional downstream structures. From this experiment, it is therefore unclear whether hippocampal projections to the lateral septum mediate the effects of hippocampal manipulations on exploratory behavior.

The dorsal hippocampus may also mediate exploratory behavior via projections to the mammillary bodies or the thalamus, which along with place cells in the hippocampus are thought to comprise a circuit involved in spatial navigation (Taube 2007). Optogenetic experiments

targeting hippocampal projections to these regions may elucidate the contributions to exploration.

4.3.3 Candidate downstream circuits through which adult hippocampal neurogenesis may affect anxiety and depression-related behavior.

The ventral hippocampus is thought to contribute to anxiety and depression-related behavior, since lesions of the ventral hippocampus have been shown to affect behavioral tests in these categories (Kjelstrup et al. 2002, McHugh et al. 2004). Furthermore, ablation of adult hippocampal neurogenesis specifically in the ventral dentate gyrus prevents some of the behavioral effects of antidepressants (Wu and Hen 2014). The ventral hippocampus sends projections to the hypothalamus, medial prefrontal cortex, nucleus accumbens and amygdala (Canteras and Swanson 1992, Namura et al. 1994). The ventral hippocampus has been hypothesized to affect anxiety and mood-related behavior through these projections (Fanselow and Dong 2010), and the behavioral effects of increasing adult hippocampal neurogenesis are therefore likely through these pathways as well.

One downstream pathway that has been hypothesized to mediate the role of adult hippocampal neurogenesis on anxiety and depression-related behavior is via disynaptic connections between the hippocampus and the paraventricular nucleus of the hypothalamus, which controls the HPA axis. As previously discussed, evidence for this hypothesis comes from ablation studies in which the absence of adult hippocampal neurogenesis alters the response of the HPA axis to stress, or the effects of antidepressants on HPA axis regulation (Schloesser et al. 2009, Snyder et al. 2011, Surget et al. 2011). However, using similar paradigms, no changes in HPA axis regulation were observed following increased adult hippocampal neurogenesis

(Figures 2.11 and 3.2). Furthermore, behavioral effects of increasing adult hippocampal neurogenesis were observed in mice treated with chronic CORT, in which the endogenous HPA axis is not responsive to stress. This suggests that increasing adult hippocampal neurogenesis in this condition most likely affects behavior through a mechanism independent of the HPA axis. Nevertheless, under different stress paradigms, increasing adult hippocampal neurogenesis may affect behavior in a HPA axis dependent manner. Future studies testing both HPA axis regulation and anxiety and depression-related behavior in mice with increased adult hippocampal neurogenesis that are exposed to additional stress paradigms will provide further mechanistic insight.

Additional ventral hippocampal projections may underlie the observed behavioral effects of increasing adult hippocampal neurogenesis on anxiety and depression-related behavior. Projections from the hippocampus to the medial prefrontal cortex have been implicated in mediating anxiety-related behavior because synchrony between these two regions increases during anxiogenic experiences (Adhikari et al. 2010, Adhikari 2014). Since the nucleus accumbens is important for reward processing, hippocampal projections to this region could be involved in anxiety and depression-related behavior (Russo and Nestler 2013). Lastly, the ventral hippocampus and the amygdala are bidirectionally connected (Pitkanen et al. 2000), and optogenetic studies have recently implicated a role for projections from the amygdala to the ventral hippocampus in anxiety and social-related behaviors (Felix-Ortiz et al. 2013, Felix-Ortiz and Tye 2014). Projections from the hippocampus to the amygdala may also contribute to these behaviors, although this has yet to be shown.

Changes in activity of hippocampal projections to various downstream regions due to increased levels of adult hippocampal neurogenesis may underlie the observed changes in

contextual fear discrimination learning, exploration, and anxiety and depression-related behavior. Future experiments utilizing optogenetics as well as techniques to observe *in vivo* activity patterns in downstream regions may indicate which downstream regions are involved in these behavioral phenotypes.

4.4 Development of novel antidepressants to target adult hippocampal neurogenesis

iMac2 is a pharmacological antagonist of BAX, which is part of the Bcl-2 family of pro-apoptotic and anti-apoptotic proteins that regulate cell death. Small molecule inhibitors of Bcl-2 (an anti-apoptotic protein in its namesake class) were initially established via structure-based computer screening methods (Enyedy et al. 2001). Subsequently, molecules with analogous structures were tested for their ability to inhibit pro-apoptotic proteins of this class based on their ability to prevent cytochrome c release through mitochondrial channels following induced apoptosis. In this way, iMac1 and iMac2 (named as inhibitors of mitochondrial apoptosis-induced channels) were discovered (Bombrun et al. 2003). Only later was BAX identified as a component of these mitochondrial apoptosis-induced channels, which can be formed by homodimers of BAX, homodimers of another Bcl-2 family protein BAK, or by heterodimers consisting of both proteins (Dejean et al. 2005). Evidence was then obtained that blocking BAX is sufficient to block apoptosis (Hetz et al. 2005). Later, iMacs were shown to suppress apoptosis through BAX antagonism, and in a screen of several small molecules, iMac2 was shown to be the most effective, in addition to having the lowest toxicity (Peixoto et al. 2009).

The potential systemic effects of iMac2-mediated antagonism of BAX were an initial concern. However, whole-life genetic deletion of *Bax* has only limited systemic effects, mainly

leading to increased weight of the spleen and thymus (Knudson et al. 1995). Here, iMac2 treatment did not significantly impact spleen or thymus weight (Figure 3.5), suggesting that iMac2 has limited effects outside of the brain. Additionally, one third of mice with deletion of both *Bax* and *Bak* in the nestin lineage leads to brain tumors (Katz et al. 2013), however no brain tumors have been reported in *Bax* knockout mice (Knudson et al. 1995), and none were observed in the experiments presented here.

Other drugs with pro-neurogenic properties have been suggested for use as antidepressants or to treat cognitive impairment. An *in vivo* screen of 1000 drugs for candidates that increase adult hippocampal neurogenesis led to the identification of P7C3 (Pieper et al. 2010). This drug increases adult hippocampal neurogenesis, improves performance in cognitive tasks in aged mice (Pieper et al. 2010), and increases social interaction time following social defeat, which has been interpreted as an antidepressant-like effect (Walker et al. 2014). Furthermore, this effect on social interaction is dependent on adult hippocampal neurogenesis, in that it is prevented in mice that have undergone hippocampal x-irradiation (Walker et al. 2014). These studies highlight P7C3 as a candidate antidepressant compound, however it is still unclear whether the observed behavioral effects are mediated solely by increased levels of adult hippocampal neurogenesis, or whether this is just one component required for behavioral effects. P7C3 has been observed to have BAX antagonist activity (D. Lorain, personal communication), but additional effects of this drug are unknown.

Two other pro-neurogenic compounds have been identified and tested. Isoxazole 9 (isx-9) is a pro-neurogenic small molecule. The precise mechanism through which isx-9 increases neurogenesis is unknown, but is likely through upregulation of the myocyte enhancer family of proteins (Mef2), since isx-9 has no effect on neurogenesis in mice with Mef2a and Mef2d

genetically deleted from adult-born neurons (Petrik et al. 2012). In addition to increasing neurogenesis, isx-9 also alters expression levels of various genes in neural stem cells, increases the dendritic complexity and soma size of adult-born neurons, and enhances performance in a spatial Morris water maze task (Petrik et al. 2012). Due to these various effects, it is unclear whether the observed behavioral effect of isx-9 is due to changes in the number of adult-born cells, changes in other properties of adult born cells, or changes in other cell types due to systemic administration of this drug. Furthermore, to our knowledge, this drug has not been tested for effects on anxiety and depression-related behavior. Another small molecule KHS101 has been identified that increases neuronal differentiation through a mechanism that promotes cell cycle exit of proliferating cells (Wurdak et al. 2010), but behavioral testing has not been performed in mice treated with this compound.

Along with these other drugs, iMac2 represents a potential novel treatment for anxiety and depression-related disorders through the mechanism of increasing adult hippocampal neurogenesis. The results presented in Chapter 3 provide the first evidence that such a drug can produce an anxiolytic effect in a model of chronic stress. It would be ideal to directly show that the observed behavioral effect of iMac2 is due to increased adult hippocampal neurogenesis. The proper way to test this would be to determine whether the behavioral effects are blocked in mice treated with iMac2 when neurogenesis is lowered to baseline levels, but we do not have a technique to do this. Performing an experiment to show that the behavioral effect of iMac2 is blocked in mice with completely ablated adult hippocampal neurogenesis (using a transgenic ablation model or hippocampal x-irradiation) would be suggestive, but could mean that some levels of neurogenesis are required for the behavioral effect, and not necessarily that increasing neurogenesis above baseline levels is important. Another control experiment would be to

administer iMac2 to CORT treated *iBax* mice with genetically increased adult hippocampal neurogenesis. If any behavioral effects of iMac2 were observed in these mice, they would be due to effects on other cells, since there would be no BAX in adult-born cells. The lack of behavioral effects in this experiment would support the interpretation that the anxiolytic effect of iMac2 reported in Chapter 3 is due to increased adult hippocampal neurogenesis.

4.5 In increasing adult hippocampal neurogenesis necessarily beneficial?

While the data presented here showcases the beneficial effects of increasing adult hippocampal neurogenesis, it should be noted that this manipulation may not be beneficial in all ways, especially if continued over a long period of time. First, as presented in the introduction, some studies have found that neurogenesis impairs performance in remote learning tasks (Saxe et al. 2007, Akers et al. 2014). Furthermore, it is possible that a larger increase in adult hippocampal neurogenesis than used here might impair pattern separation. This is suggested by the finding that increasing the number of adult-born cells in the olfactory bulb impairs performance in odor discrimination tasks (Mouret et al. 2009). Optimal memory processing requires a delicate balance between pattern separation and pattern completion, therefore tilting this balance too far in either direction could be detrimental.

Second, the generation of adult-born neurons requires energy. Decreasing adult-hippocampal neurogenesis when exposed to stress may be an adaptive mechanism in order to delegate energy to more important processes. Furthermore, one study suggests that adult hippocampal neurogenesis generates oxidative stress, which has been implicated in contributing to various neurologic and psychiatric diseases (Walton et al. 2012). Increasing adult hippocampal

neurogenesis may therefore be maladaptive in ways that may be observed over longer periods of time than tested here.

4.6 Conclusion

The work presented in this thesis shows that increasing adult hippocampal neurogenesis differentially impacts cognitive, exploratory, and anxiety and depression-related behavior under baseline, voluntary exercise or chronic CORT conditions. These dissociated effects may be due to different mechanisms within the hippocampus, or mediated by different downstream circuits. Expansion of adult hippocampal neurogenesis using small molecule drugs such as iMac2 represent potential novel therapeutics for cognitive impairment as well as depression and anxiety disorders. Since these disorders represent heterogeneous patient populations, drugs that target adult hippocampal neurogenesis may be especially useful to treat patients with dentate gyrus dysfunction, identified through imaging or behavioral tests that are currently being developed.

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Appendix A: Increasing adult hippocampal neurogenesis after the onset of chronic CORT treatment

As presented in Chapter 3, increasing adult hippocampal neurogenesis *before* the onset of chronic CORT treatment is sufficient to prevent the effects of CORT on anxiety and depression-related behavior. However, a more translationally relevant experiment is to increase adult hippocampal neurogenesis *after* the onset of CORT. After three weeks of CORT treatment, a length of time that is sufficient for CORT to affect behavior (David et al. 2009), *iBax* mice were administered either tamoxifen (TAM) or vehicle, and tested on various behavioral tests six weeks later (Figure A.1 a). (All experimental methods were conducted as described in Section 3.2.)

Immunostaining for DCX and BrdU was performed in order to assess levels of adult hippocampal neurogenesis (Figure A.1 b,c). In this experiment, there was no effect of CORT treatment on the total number of cells labeled with DCX ($F(1,6)=0.137$, $p>0.05$), or the number of DCX-positive cells with tertiary dendrites ($F(1,6)=0.110$, $p>0.05$) (Figure A.1 d). However, in mice treated with chronic CORT, TAM treatment induced an increase in both the total number of DCX-positive cells ($F(1,6)=8.468$, $p<0.05$), as well as the number of DCX-positive cells with tertiary dendrites ($F(1,6)=10.927$, $p<0.05$). There was no significant effect of CORT ($F(1,9)=0.155$, $p>0.05$) or TAM ($F(1,8)=1.242$, $p>0.05$) for the number of BrdU-positive cells.

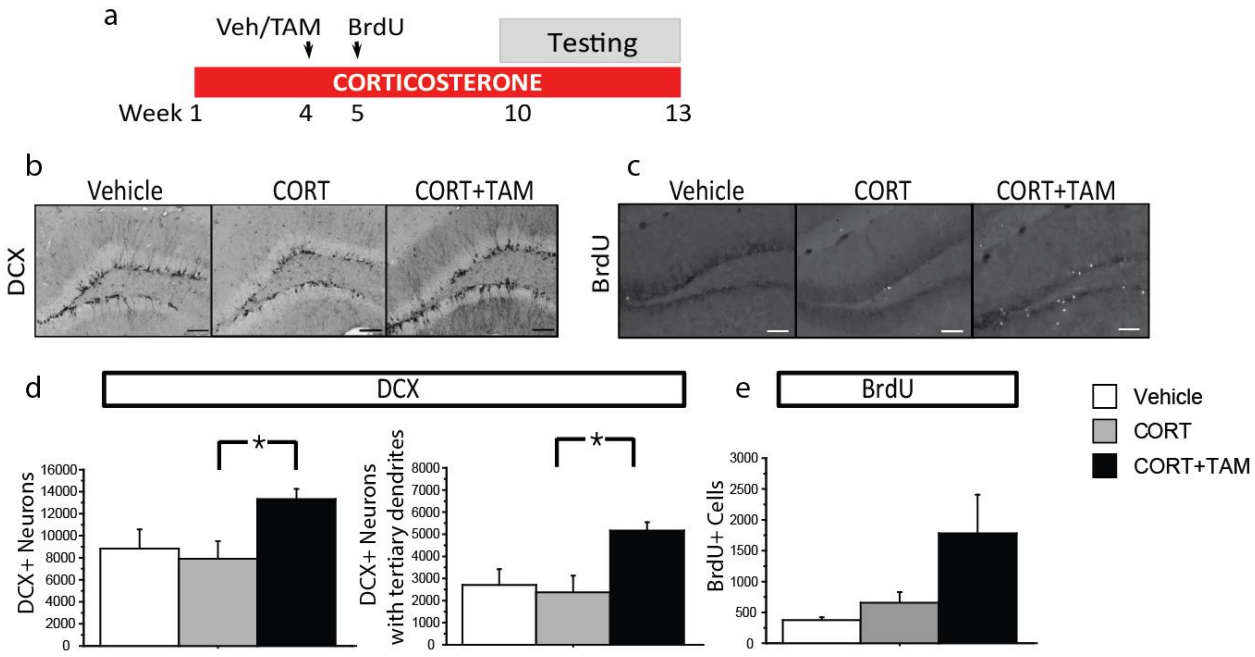

Figure A.1 TAM treatment increases neurogenesis when administered to *iBax* mice three weeks into chronic CORT treatment.
a) Experimental design. b-c) Representative images of DCX and BrdU (scale bars 100 um). d) CORT treatment has no effect on DCX-positive neurons (p=0.724) or the number of DCX-positive neurons with tertiary dendrites (p=0.751). CORT+TAM treated mice have significantly higher numbers of DCX-positive neurons (p=0.027) and DCX-positive neurons with tertiary dendrites (p=0.016), as compared to mice treated with CORT alone. e) There are no significant effects of either CORT (p=0.703) or TAM (p=0.297). n=4-8 mice/group. All error bars represent SEM. *p<0.05.

Mice were tested to determine whether increasing adult hippocampal neurogenesis is sufficient to rescue the behavioral effects of chronic CORT. Here, we observed no behavioral effects of CORT or TAM in the open field, forced swim or tail suspension tests (Figure A.2 a,c,d). However, in the elevated plus maze, we observed trends for both an effect of CORT compared to vehicle treated animals ($F(1,11)=3.718$, $p=0.08$), and an effect of CORT+TAM compared to animals treated with CORT alone ($F(1,11)=3.698$, $p=0.08$).

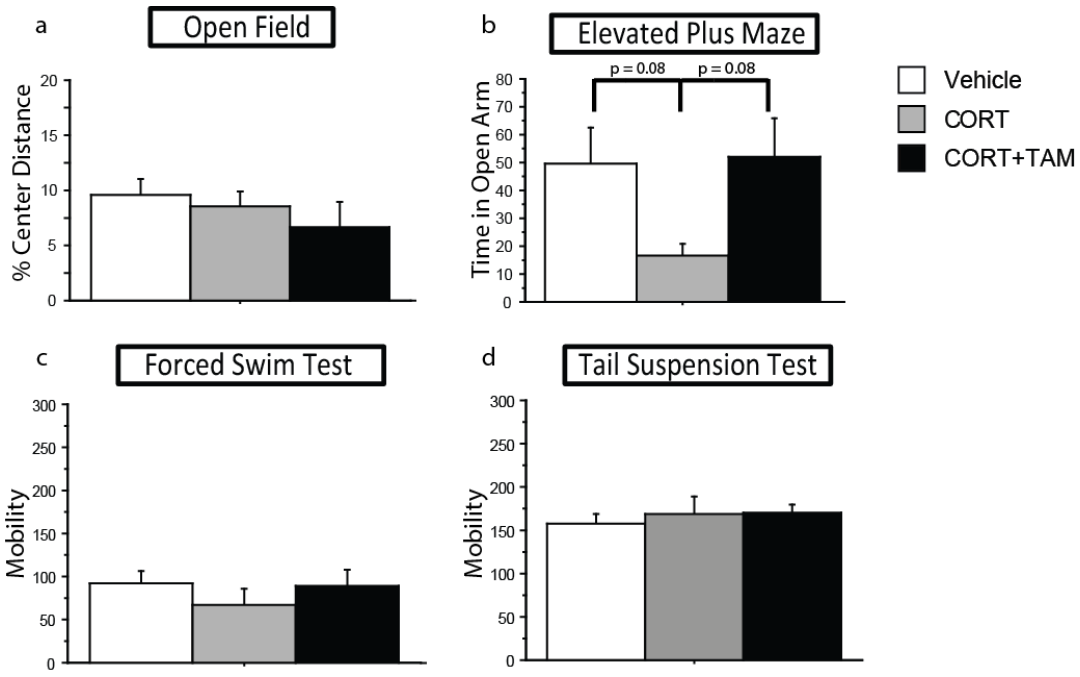

Figure A.2 Increasing adult hippocampal neurogenesis in *iBax* mice is sufficient to rescue the anxiogenic effect of CORT in the elevated plus maze.
a) There is no effect of CORT (p=0.642) or TAM (p=0.572) in percent center distance in the open field test. b) In the elevated plus maze, there is a trend for decreased time in the open arms in CORT treated mice (p=0.08), and a trend for this effect to be rescued in mice treated with chronic CORT (p=0.08). c) There is no effect of CORT (p=0.346) or TAM (p=0.497) in mobility in the second day of the forced swim test. d) There is no effect of CORT (p=0.670) or TAM (p=0.888) in mobility in the tail suspension test. n=4-8 mice/group. All error bars represent SEM.

This result provides preliminary evidence that increasing adult hippocampal neurogenesis after the onset of a chronic stressor may be sufficient to rescue the behavioral effects of stress. Follow up studies with additional stress paradigms, as well as using pharmacological agents to increase adult hippocampal neurogenesis, such as iMac2, will provide more insight into this hypothesis.

Appendix B: Optogenetic stimulation of hippocampal projections to the lateral septum

B.1 Introduction

Many animal studies as well as patient case studies have implicated a role for the hippocampus in mediating many behaviors, including memory, exploratory and mood-related behavior. However, it is unclear which downstream projection regions from the hippocampus mediate these behavioral effects. Optogenetics has allowed exploration of this question, through the activation or silencing of hippocampal terminals in specific downstream brain regions.

Channelrhodopsin-2 (ChR2) is a light gated cation channel that can be functionally expressed in mammalian cells (Nagel et al. 2003). Illumination with 473 nm light opens ChR2, allowing an influx of sodium ions, and a depolarization that can be propagated along axons and lead to neurotransmitter release (Boyden et al. 2005, Zhang et al. 2006). Additionally, axonal terminals with ChR2 expression can be directly illuminated to selectively activate projections from one brain region to another (Tye and Deisseroth 2012).

Here, we wished to determine the behavioral consequences of specifically activating hippocampal projections to the lateral septum using ChR2.

B.2 Methods

B.2.1 Mice

Experiments were performed using male 129SvEv mice ordered from Taconic. Mice were housed 2-5 per cage and maintained on a 12 hour light/dark schedule with continuous

access to food and water. All behavioral testing was conducted during the light cycle with approval from the Institutional Animal Care and Use Committees at both Columbia University and the New York State Psychiatric Institute.

B.2.2 Viral injection and chronic implantation of fiber optic

Mice were anesthetized with sodium pentobarbital (7 mg/kg) and placed in a stereotaxic frame. A 26-Gauge Hamilton syringe (Model 701) was placed above the hippocampus (coordinates from Bregma: 3.2 ML, -3.0 AP, -3.7 DV), and lowered using a microinjector at a rate of 0.5 mm/min. 1 µL of virus was injected into the right hippocampus at a rate of 0.1 µl/min. The syringe was left in place for 5 minutes following injection to allow for diffusion of viral particles. The syringe was then removed at a rate of 0.5 mm/min. Mice were injected with either pAAV5-hSyn-EYFP (referred to as EYFP) or pAAV5-hSyn-hChR2(H134R)-EYFP (referred to as ChR2-EYFP) virus (titer 4x10^12 virus molecules/ml), obtained from the University of North Carolina viral vector core.

During the same surgery, a fiber optic was chronically implanted into the right lateral septum (coordinates from Bregma: 0.8 ML, +0.2 AP, -2.6 DV), and secured to the skull with dental cement (Dentsply, 675570). Fiber optics were constructed similarly to as has been described (Sparta et al. 2012), using fiber with a 200 µm core and 0.37 numerical aperture (ThorLabs), which was threaded through and glued into a stainless steel ferrule. On one end of the ferrule, a 3 mm length of fiber extended for penetration of the brain to the lateral septum. On the other end of the ferrule, excess fiber was cut with a diamond pen, and polished, for connection to a patch cable during behavioral testing. Ferrules were tested for light output

following construction, and all fibers used had output >75%. After surgery, mice were returned to their home cage and monitored until recovery.

3.2.3 Behavioral testing

Behavioral testing was conducted 6 weeks after viral surgery to allow for expression and transport of ChR2 to the axon terminals. In order to habituate mice to the behavioral procedure, they were handled and attached to a patch-cord for 5 minutes on each of the two days prior to testing. Patch cables were constructed as described (Sparta et al. 2012), using 200 µm diameter fiber.

During the behavioral experiment, mice were attached to a patch-cord, which was connected to a 1x1 rotary joint optical commutator to prevent twisting of the patch cord during locomotion. On the other end, the commutator was coupled to a solid-state 473 nm, 100 mW laser (OEM Laser Systems, Inc.). The laser was controlled by a Master-8 stimulator (A.M.P.I.), which was programmed to deliver 10 msec duration light pulses at 10 hertz, a frequency within the typical firing range observed for hippocampal output cells (Sharp and Green 1994, Hemond et al. 2008). Due to the high ChR2 expression levels observed in pilot experiments, light was delivered at low power, <1 mW.

Mice were placed in an open field (MED Associates) for a total of 15 minutes, which was split into 5 minute epochs. During the first and third epoch, there was no laser light allowed through the patch cord to the mouse (‘light OFF’ epochs). During the middle ‘light ON’ epoch, mice received fiber optic illumination. Behavioral measures were analyzed using MED Associates software. Animals were carefully observed throughout behavioral testing. Two

animals displayed seizure-like behavior. They were immediately removed from the behavioral test, and data from these mice were not analyzed.

B.2.4 Immunohistochemistry

At sacrifice, mice were anesthetized with ketamine and xylazine (100 and 7 mg/kg respectively). Mice were then transcardially perfused with cold saline and 4% paraformaldehyde. Brains were postfixed in 4% paraformaldehyde overnight, and then transferred to 30% sucrose for cryoprotection. Brains were cut coronally on a cryostat into 35 µm sections.

Staining for GFP and cFos (or GFP alone) was performed on floating sections. Tissue was first washed in phosphate-buffered saline with .1% triton-X (PBST) 3 times for 10 minutes each. Sections were then blocked in PBST with 10% normal donkey serum for 2 hours a room temperature, and placed in primary antibodies overnight at 4°C (rabbit anti-cFos 1:5000 [EMD Millipore, PC38], chicken anti-GFP 1:500 [Abcam, ab13970] in blocking solution). The following day, sections were washed in PBS 3 times for 10 minutes each, placed in secondary antibodies for 2 hours at room temperature (Donkey anti-rabbit Cy3 1:250, biotinylated donkey anti-chicken 1:250 [Jackson ImmunoReasearch] in PBS), washed in PBS for 3 times 10 minutes each, placed in tertiary antibody for 1 hour at room temperature (Avidin-Cy2, 1:250 [Jackson ImmunoResearch] in PBS), washed in PBS 3 times 10 minutes each, and mounted onto slides. Imaging was performed using an Olympus Fluoview confocal microscope.

B.2.7 Statistical methods

Repeated measures ANOVA was used to assess the interaction between treatment and time over the three time bins in the open field test, followed by post-hoc tests where appropriate.

Statistical analysis was conducted using StatView software (SAS Institute, Cary, NC). Results were considered statistically significant if $p < 0.05$.

B.3 Results and discussion

In order to determine the functional capability of hippocampal projections to the lateral septum, a virus encoding ChR2 (CHR2-EYFP), was unilaterally injected into the right hippocampus (Figure B.1). In the virus used, ChR2 is fused to EYFP for visualization, and is expressed under the human Synapsin (hSyn) promoter, which allows expression in all neuronal cell types (Kugler et al. 2003). In mice injected with this virus, ChR2-EYFP expression can be assessed using immunostaining for GFP. Such staining indicated that the virus is incorporated into neuronal cells in the hippocampus: expression is highest in CA3, but also present in CA1 and the dentate gyrus.

In neurons that express ChR2, this channel is transported to long-range axonal terminals over several weeks (Stuber et al. 2011). Here, ChR2-EYFP expression can be seen in terminal projections 6 weeks after viral injection. Labeled terminals were observed in prefrontal cortex (PFC), lateral septum (LS), and bed nucleus of the stria terminalis (BNST) (Figure B.1), as well as in the amygdala and hypothalamus (not shown).

The most densely labeled terminals were observed in the lateral septum. This is likely because viral incorporation is most dense in CA3, and the lateral septum is the only extrahippocampal projection region of CA3 (Swanson and Cowan 1977, Witter 2007). Projections observed in the lateral septum may be from cells in CA3, CA1 or subiculum, while labeled terminals in other extrahippocampal regions are only from CA1 and subiculum. CA3 projections are bilateral, while projections from CA1 and the subiculum are unilateral (Swanson

and Cowan 1977), leading to the observed bilateral labeling in the lateral septum, and unilateral labeling in the PFC and BNST (Figure B.1).

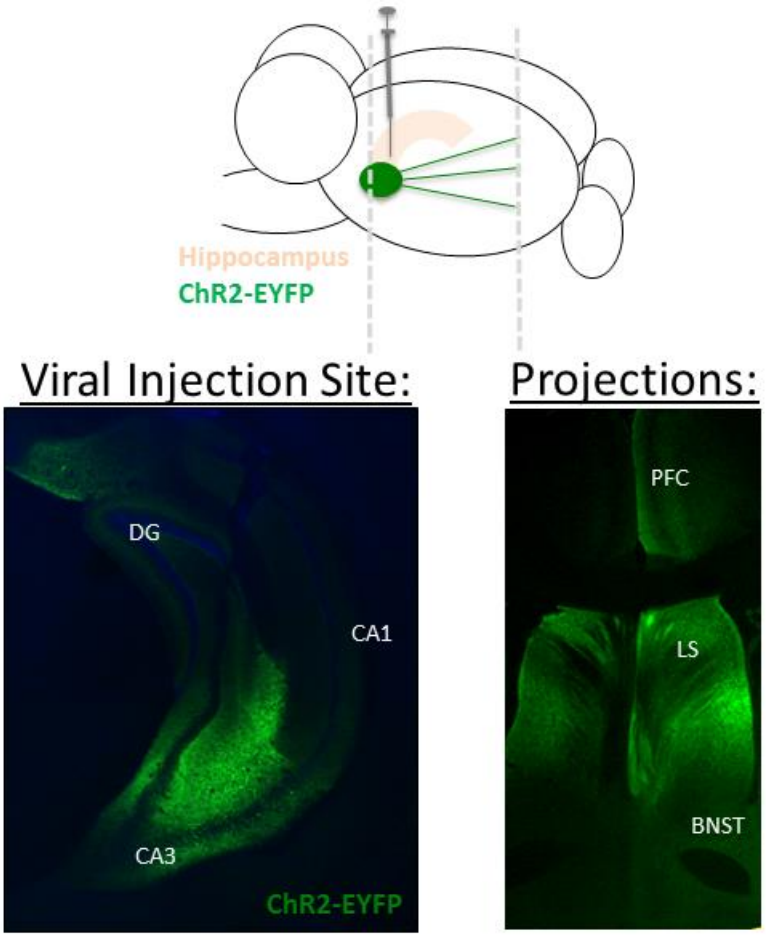

Figure B.1 Viral expression of ChR2-EYFP injected into the right hippocampus.
ChR2 was injected into the right hippocampus. Expression can be seen primarily in CA3, as well as in the dentate gyrus (DG) and CA1. ChR2 expressing terminals are observed in the prefrontal cortex (PFC), lateral septum (LS), and bed nucleus of the stria terminalis (BNST).

Six weeks after viral injection and fiber optic implantation, mice were tested in the open field test for effects of laser stimulation on exploratory and anxiety-related behavior. Here we observed a significant interaction between treatment and time for the total distance traveled ($F(2,24)=3.812$, $p<0.05$) (Figure B.2 a), where ChR2-EYFP mice displayed increased total

distance traveled during the 'light ON' epoch ($F(1,12)=5.585$, $p<0.05$). There were no significant interactions between treatment and time for percent center distance ($F(2,24)=0.67$, $p>0.05$), time in center ($F(2,24)=0.291$, $p>0.05$), or rearing behavior ($F(2,24)=0.553$, $p>0.05$) (Figure B.2 b-d).

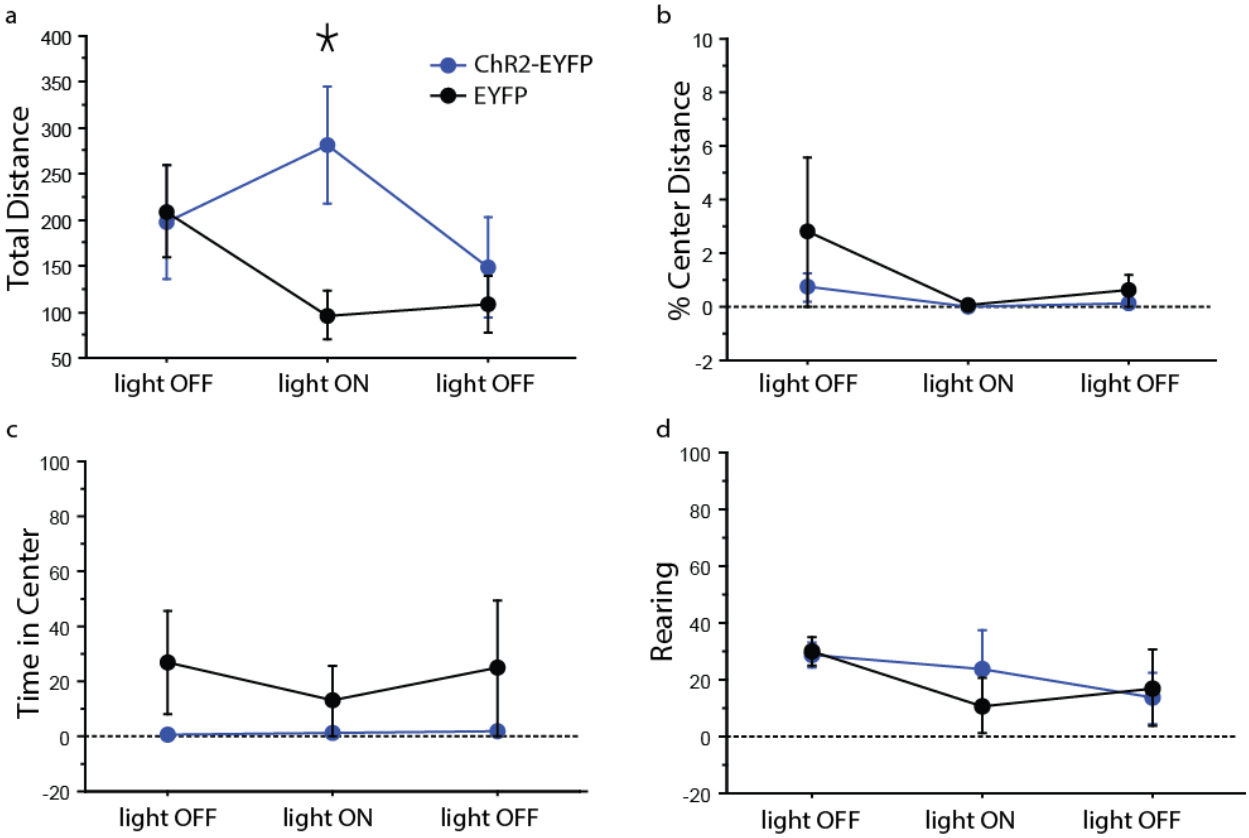

Figure B.2 Stimulation of ChR2 expressing hippocampal terminals in the lateral septum increases total distance traveled in the open field.
a) In the open field test, there is a significant interaction between treatment and time for total distance traveled ($p=0.037$). ChR2-EYFP mice display increased total distance traveled during the light on epoch as compared to EYFP mice ($p=0.036$). b-d) There is no significant interaction between treatment and time for percent center distance ($p=0.521$), time in center ($p=0.750$) or rearing ($p=0.582$). n=6-8/group. Results are presented as mean ± SEM. * $p<0.05$.

Mice were sacrificed 90 minutes after testing in the open field in order to assess activity levels using immunostaining for cFos. Here we observed that mice expressing ChR2-EYFP

display dense cFos labeling in the hippocampus, particularly in the dentate gyrus, while mice expressing EYFP display sparse cFos activity (Figure B.3).

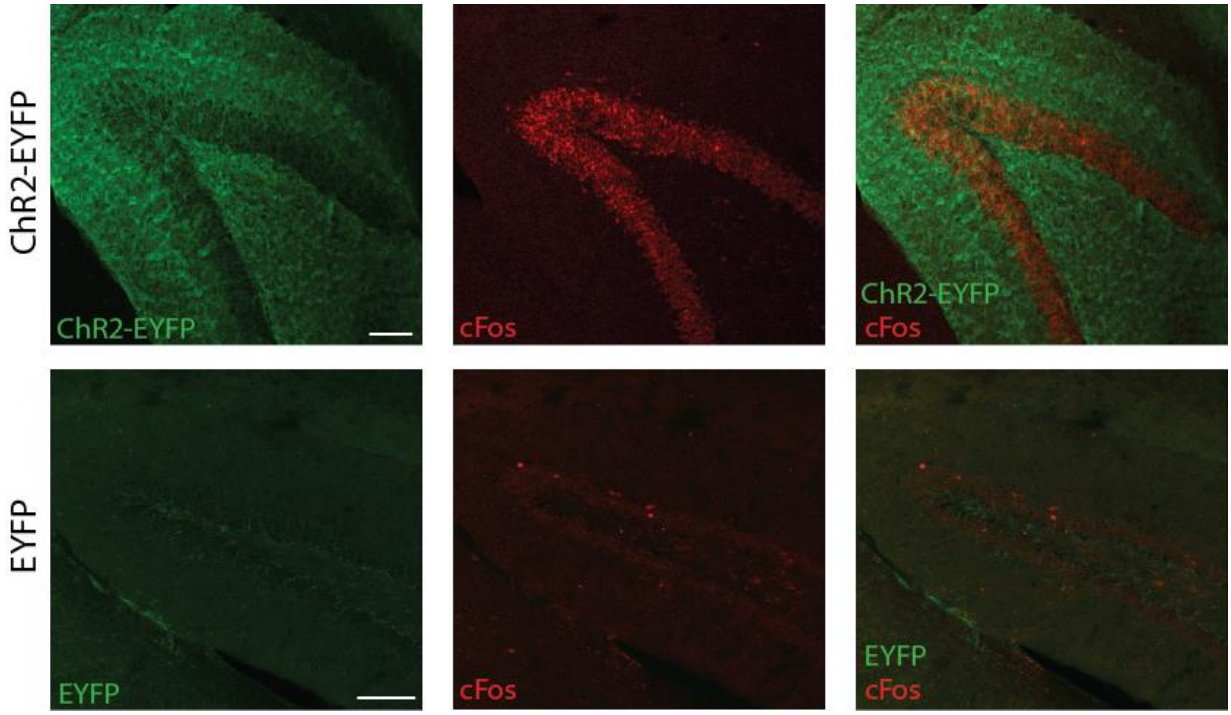

Figure B.3 Stimulation of hippocampal ChR2-EYFP terminals in the lateral septum induces high levels of cFos staining in the hippocampus.
Images of sections from ChR2-EYFP (top row) and EYFP (bottom row) mice stained for EYFP (left column) cFos (middle column) and merged (right column). Scale bar 100 µm.

ChR2 excitation at terminals can lead to anterograde action potentials that can activate cell bodies. However, since dentate granule cells do not themselves project to the lateral septum (or anywhere outside of the hippocampus), the observed cFos expression could not be due to direct activation of dentate granule ChR2 expressing terminals. Dentate gyrus activation may be due to feed forward circuits through the lateral and medial septum. Since the lateral septum sends inhibitory projections to the medial septum (Swanson and Cowan 1979), and the medial septum sends inhibitory projections to the dentate gyrus (Kohler et al. 1984), stimulation of hippocampal

terminals in the lateral septum may disinhibit dentate granule cells, increasing activation. Alternatively, the observed cFos activation in the dentate gyrus may be a downstream result of hippocampal activation due to anterograde activation of CA3 and CA1 pyramidal cells whose terminals are activated in the lateral septum. Many single CA3 pyramidal cells have been observed to send projections to both CA1 and the lateral septum (Swanson et al. 1980), thus anterograde activation of these cell bodies would be likely to activate CA1, which could activate the entire hippocampus via circuits through the entorhinal cortex.

If the exploration phenotype observed here is due to stimulation of hippocampal projections in the lateral septum, this behavior may be mediated by projections from the lateral septum to the ventral tegmental area (VTA) (Swanson and Cowan 1979), which likely disinhibits dopaminergic cells located there (Luo et al. 2011). Since dopamine is involved in exploratory behavior (Smith 1976), the hippocampus-lateral septum-VTA is a candidate circuit for mediating hippocampal modulation of exploratory behavior.

In addition to determining whether hippocampal projections to the lateral septum do indeed mediate the observed exploratory phenotype, future studies will also shed light on whether this projection mediates hippocampal modulation of other behaviors. The hippocampus and VTA have also been hypothesized to form a loop that is involved in the formation of long-term memory (Lisman and Grace 2005). In addition to the VTA, the lateral septum projects to various hypothalamic areas, the raphe nucleus, amygdala and thalamus (Meibach and Siegel 1977, Swanson and Cowan 1979). The lateral septum has also been implicated in playing a role in social behavior (Ophir et al. 2009), anxiety (Le Merrer et al. 2006, Singewald et al. 2011, Trent and Menard 2011), memory (Reis et al. 2010) and reward (Luo et al. 2011). The

hippocampal projection to the lateral septum therefore has the potential to modulate various behaviors.

Appendix C: Generation of a transgenic mouse line to target the ventral, posterior hippocampus

C.1 Introduction

As introduced in Chapter 1, much work has differentiated the dorsal and ventral subregions of the hippocampus based on anatomical connectivity, gene expression and contribution to cognitive and mood-related behavior (Bannerman et al. 2004, Fanselow and Dong 2010). While in previous studies subregions along this axis have been targeted stereotaxically, genetic tools are needed for more precise targeting. Here, we present a transgenic tool that can be used to target the ventral, posterior hippocampus.

In a microarray screen for genes with different expression levels in the dorsal and ventral regions of the hippocampus, decorin was identified as one of the proteins most enriched in the ventral hippocampus, with over 11 fold increased expression in the ventral over dorsal hippocampus (Leonardo et al. 2006). The microarray finding was validated with in situ hybridization, showing decorin expression in ventral, but not dorsal, CA1 and subiculum (Leonardo et al. 2006).

Decorin is a small, leucine-rich proteoglycan, primarily studied for its involvement in regulating matrix assembly (Iozzo 1999), along with cell adhesion, growth, migration (Ferdous et al. 2010), and possibly neurotrophin signaling (Sometani et al. 2001). Neuronal expression outside of the hippocampus has not yet been characterized; however a transgenic mouse line was previously made using the decorin regulatory elements from a bacterial artificial chromosome (BAC), where neuronal transgene expression was confined to the ventral hippocampus (C.

Gross, personal communication). There is also evidence for decorin expression outside of the brain, including in muscle, liver and endothelial cells (Brandan et al. 1991, Zanotti et al. 2005).

BACs typically can contain segments of DNA ranging from 100 to 300 kilobases (Shizuya et al. 1992). A BAC containing a gene of interest therefore can encompass not only the full coding region, but also many, if not all, cis-regulatory elements that govern the timing and location of expression (Muyrers et al. 2001). Recombineering techniques have been developed to manipulate DNA in BACs, allowing for efficient insertion of transgenes into BACs (Copeland et al. 2001). When BACs are inserted into the genome, they insert randomly. However, due to the large size of BACs, expression of genes within a BAC is often not affected by insertion site (Gong et al. 2003).

Here we describe the generation of a transgenic mouse line, using a BAC to express the inducible recombinase CreERT2 under the decorin promoter and regulatory elements (DCN:CreERT2). Mice hemizygous for DCN:CreERT2 as well as a Cre-dependent tdTomato reporter line (Ai9 (Madisen et al. 2010)) express tdTomato in principal cells of the ventral, posterior hippocampus. We anticipate that this will be a useful genetic tool to target deletion of various genes specifically form the ventral, posterior hippocampus, as well as a means to target projections from this region throughout the brain.

C.2 Methods

C.2.1 Generation of transgenic mice

A BAC clone containing the *decorin* gene (RP24-286G12) was obtained from the BACPAC Resource Center (Children's Hospital Oakland Research Institute). The experimental design for insertion of CreERT2 into this BAC is depicted in Figure C.1. First, the BAC was

electroporated into SW102 cells, a tetracycline resistant strain that contains a temperature-inducible prophage that can mediate homologous recombination (obtained from the National Cancer Institute (Warming et al. 2005, Sharan et al. 2009)) (Figure C.1 Step 1). Next, a loxP site that is present in this BAC was replaced so that it would not interfere with future loxP mediated recombination. This was accomplished using homologous recombination to insert a cassette containing a Zeocin resistance gene, which was used for selection of recombined clones (p24loxZeo cassette, a generous gift from Dr. Kosuke Yusa, Wellcome Trust Sanger Institute) (Figure C.1 Step 2). Next, the cassette to insert CreERT2 into the BAC was generated, and inserted into the BAC, replacing the decorin start site (Figure C.1 Step 3).

Generation of the CreERT2 cassette is described in Figure C.2. Primers (Integrated DNA Technologies) were designed to amplify CreERT2 between the upstream BAC homology arm on one side, and a SalI restriction site on the other side. Using these primers, CreERT2 was amplified (from a DNA fragment provided as a gift from P. Chambom), and the PCR product was cloned using the TOPO PCR cloning method (Life Technologies). Next, PCR was used to amplify a kanamycin resistance gene using primers that also contained FRT sites, as well as a Sal1 site upstream, and the downstream BAC homology sequence in the reverse primer. This PCR product was also TOPO cloned. Next, these two PCR products were digested out of the TOPO vectors, and ligated via Sal1 restriction sites. This cassette was used to replace the decorin transcription start site in the BAC with CreERT2 via homologous recombination, and positive clones were selected by resistance to kanamycin (Figure C.1 Step 3).

The recombined BAC was then electroporated into SW105 cells, which contain an arabinose-inducible *flpe* gene (Warming et al. 2005) (Figure C.1 Step 4). Cells containing the recombined BAC were grown in Luria Broth (LB) containing .1% L-arabinose for 1 hour (Lee et

al. 2001) and then screened for kanamycin sensitivity (Figure C.1 Step 5). A positive clone from this screening was verified via PCR and sequencing (Genewiz), and used to generate the BAC transgenic mouse.

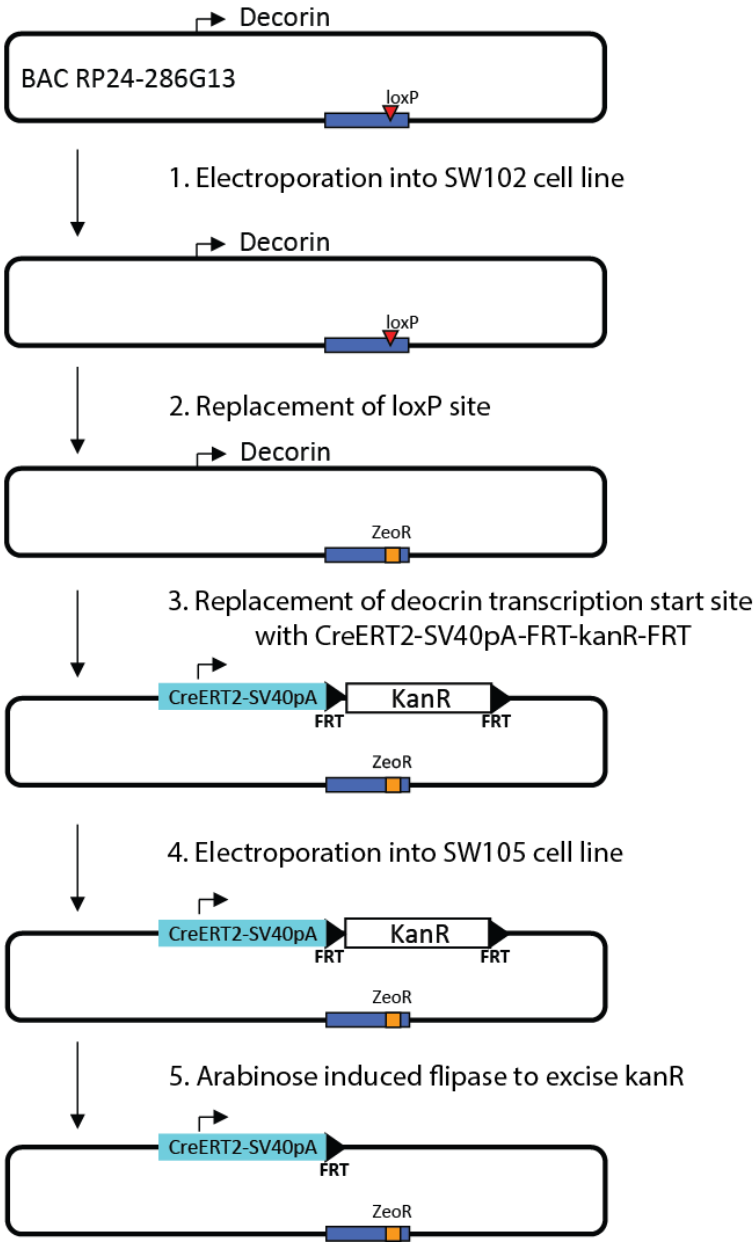

Figure C.1 Strategy to insert CreERT2 into the Decorin BAC.

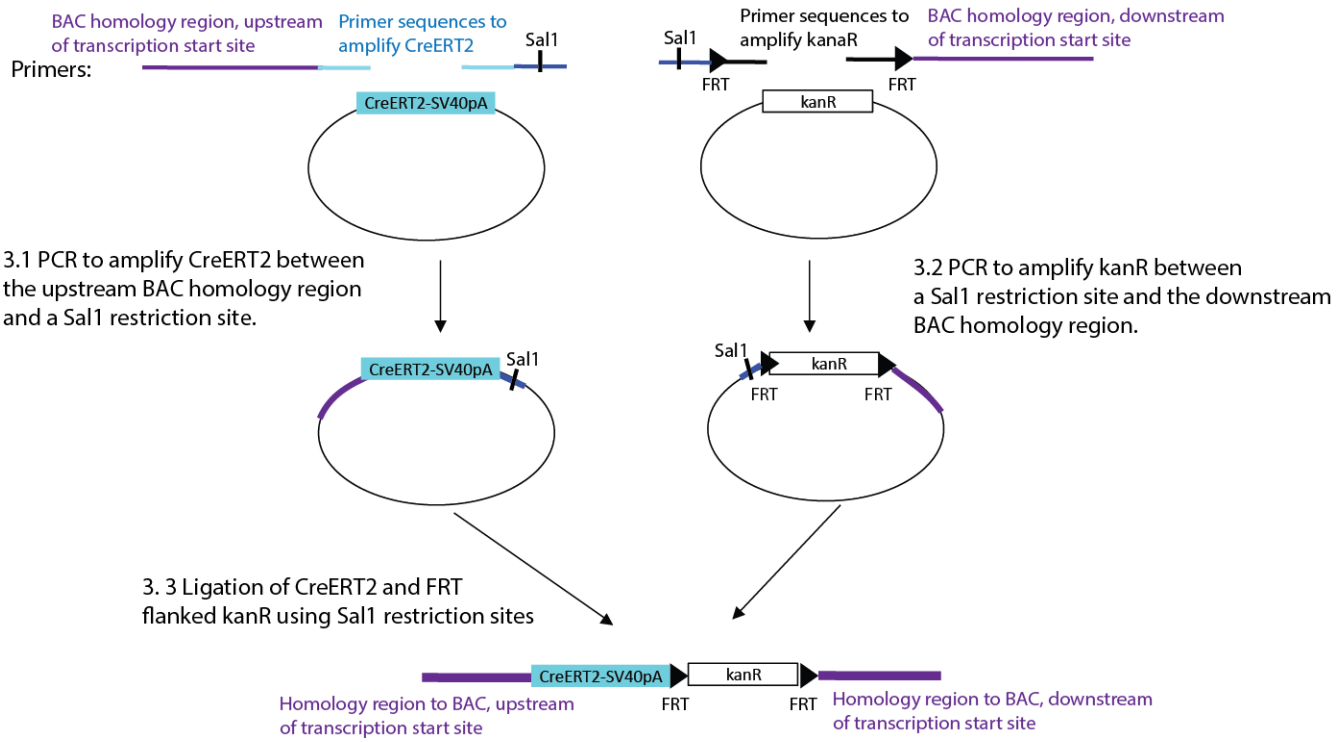

Figure C.2 Strategy to generate construct containing CreERT2 for insertion into Decorin BAC.

To generate transgenic mice, the Dcn:CreERT2 BAC DNA was linearized with Fse1 and sent to the Hochgeschwender laboratory at Duke University. There, the DNA was injected into fertilized eggs, which were then implanted into pseudopregnant female mice (Cho et al. 2009). From this procedure, three founders were generated that transmitted CreERT2 expression through the germline to offspring.

C.2.2 Breeding

F1 CreERT2-positive offspring were bred to the Ai9 tdTomato Cre reporter line (The Jackson Laboratory) to generate bi-transgenic mice, hemizygous for both DCN:CreERT2 and

Ai9. This reporter line was chosen due to the cre-dependent bright labeling of cell bodies, as well as visualization of labeled processes (Madisen et al. 2010).

Genotyping was performed using a designated set of primers for this transgenic line. The forward primer is located in the BAC (TGC TAC TGG CAA GGA AAT G), while the reverse primer (TAG CGC CGT AAATCA ATC G) is located within CreERT2. Samples containing CreERT2 display a band at 1064 base pairs. Genotyping for CreERT2 in this line was initially also confirmed using the Jackson Laboratories 'generic cre' PCR protocol.

C.2.3 Tamoxifen administration, sacrifice and tissue processing

F2 offspring hemizygous for both DCN:CreERT2 and the Ai9 tdTomato reporter transgene were used to characterize the DCN:CreERT2 line. These mice were administered tamoxifen (2 mg/kg/day) i.p. for 5 consecutive days, and sacrificed 6 weeks after the last tamoxifen treatment via transcardial perfusion. Brains were post-fixed, cryoprotected and cut into 35 µm sections. Sections were mounted onto slides for visualization of tdTomato, which does not require immunohistochemistry.

C.3 Results and discussion

Dcn:CreERT2 transgenic mice were generated as described in the methods section. A notable divergence from traditional recombineering protocols was the use of a kanamycin resistance cassette flanked by FRT sites for selection of integration of CreERT2 into the BAC. The kanamycin resistance cassette was then easily removed by transferring the BAC into a readily available cell line that has arabinose-induced flippase activity. This novel recombineering technique replaced a difficult homologous recombination step with a simple protocol. The

homologous recombination step that this replaces typically has a success rate of about 1% of screened clones (Copeland et al. 2001), while here, this novel step had a success rate of 98% of positive screened clones. Furthermore, a plasmid was generated containing CreERT2 and the FRT site flanked kanamycin resistance cassette, which can now be easily modified with homology arms for inserting CreERT2 into the coding region of a BAC containing any gene of interest.

Mice expressing DCN:CreERT2 were crossed to the Ai9 Cre-inducible tdTomato transgenic mouse line. Offspring were administered tamoxifen for inducible activation of Cre recombinase and sacrificed six weeks later. Of the three founder lines, one line has more robust expression than the others, and therefore is presented here. Interestingly, in this line, when a CreERT2-positive F1 male was used for breeding, 100% (29/29) of female F2 offspring were found to be CreERT2 positive, while 0% (12/12) of male F2 offspring were found to be CreERT2 positive, suggesting that the CreERT2 is likely inserted into the X chromosome, therefore only being passed to the female offspring of a male carrier.

Brains from F2 offspring were imaged for tdTomato expression. TdTomato is localized predominantly in the cell bodies, but can also be visualized in processes. In DCN:CreERT2;Ai9 mice, dense expression of tdTomato is seen in the posterior/ventral hippocampus, with some expression in CA1, and dense expression in the subiculum, parasubiculum and medial entorhinal cortex (Figure C.3 g-i). Sparser expression in also observed in the dorsal posterior subiculum (Figure C.3 g). Low levels of ectopic expression are present, but restricted to cingulate cortex, in a much sparser pattern than in the ventral, posterior hippocampus (Figure C.3, c compared to g,h,i).

Terminals within the hippocampus are seen in the middle third of the molecular layer of the dentate gyrus, and the stratum lacunosum-moleculare of CA3 and CA1. These are most likely terminals of projections from the medial entorhinal cortex, which has been reported to project to these layers (van Groen et al. 2003).

Outside of the hippocampus, labeled terminals are visualized in the anterior olfactory nucleus (Figure C.3 a), nucleus accumbens (Figure C.3 b,c), cingulate cortex (Figure C.3 c,d), lateral septum (Figure C.3 c,d,e), bed nucleus of the stria terminalis (Figure C.3 d,e), amygdala (Figure C.3 f) and hypothalamus (Figure C.3 f). These are the projection regions that have been previously reported for the ventral hippocampus following localized dye and viral injections (Swanson and Cowan 1977, Canteras and Swanson 1992, Kishi et al. 2000). In all brain sections, additional staining of elongated structures is seen, which is likely due to expression of tdTomato in endothelial cells of blood vessels (Figure C.3), although the identity of these cells have not been confirmed. Expression outside of the brain has not yet been assessed.

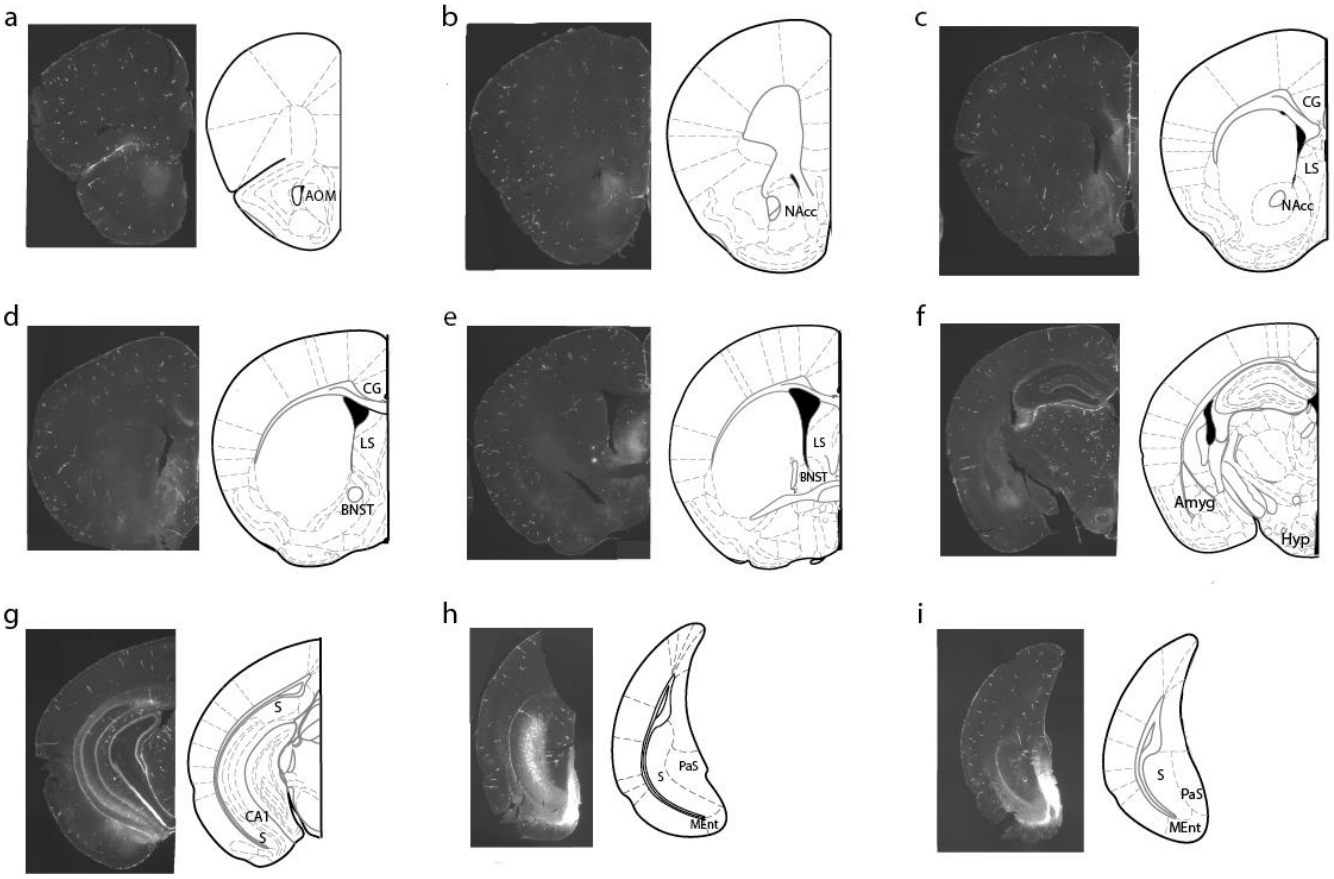

Figure C.3 TdTomato expression under Dcn:CreERT2.
Images of tdTomato expression in Dcn:CreERT2;Ai9 mice. Expression can be seen in cell bodies of ventral, posterior CA1 (g), subiculum (g,h,I; S), parasubiculum (h,I; PaS) and medial entorhinal cortex (h,I; MEnt), with no expression in principal cells of the dorsal hippocampus (f). Projections of labelled cells can be visualized in the anterior olfactory nucleus (a; AOM), nucleus accumbens (b,c; NAcc), cingulate cortex (c,d; CG), lateral septum (c,d,e; LS), bed nucleus of the stria terminalis (d,e; BNST), amygdala (f; Amyg) and hypothalamus (f; Hyp). Atlas images adapted from (Paxinos and Franklin 2001).

This mouse line is the first transgenic tool to allow manipulation of various genes and projections from the ventral hippocampus. We anticipate this it will be very useful in continuing exploration of the function of this hippocampal region.

Appendix References

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