Role Of Hippocampal Neurogenesis In The Etiology And Treatment Of Mood & Anxiety Disorders

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Abstract
Mood disorders are associated with deficits in hippocampal size and function. Neurogenesis in the dentate gyrus of the hippocampus has been implicated in the behavioral efficacy of some antidepressants. However, the precise function of newborn hippocampal neurons in the etiology and treatment of mood disorders remains unclear. Anxiety, which is highly comorbid with mood disorders, also appears to be influenced by neurogenesis. The intricacies of this influence are poorly understood. Here, the role of neurogenesis in the pathophysiology and treatment of mood and anxiety disorders is investigated in a transgenic mouse exhibiting partial suppression of hippocampal neurogenesis. This reduction is induced by hippocampal microinjection of a Cre-expressing adeno-associated virus to delete ATR, a cell cycle checkpoint kinase, from the hippocampus of adult mice. Subsequent to hippocampal ATR deletion, behavior is examined in mouse models of anxiety, depressive-like behavior, antidepressant efficacy, and susceptibility to stress. In addition, the necessity of heightened neurogenesis in exercise-induced anxiety is examined. ATR deletion resulted in an anhedonic phenotype in a sucrose drinking task but did not increase the likelihood of developing behavioral despair in the learned helplessness paradigm. Furthermore, mice lacking hippocampal ATR exhibited reduced anxiety in a number of behavioral tests. In the novelty-induced hypophagia task, the ability of chronic antidepressants to alleviate hyponeophagia was absent in ATR-deleted mice, and this effect was linked to an attenuation of the ability of chronic antidepressants to stimulate dendritic spine density. Finally, ATR deletion attenuated the neurogenic effect of voluntary wheel running, and concurrently blocked some of the anxiogenic effects of wheel running. In conclusion, neurogenesis appears to be an essential regulator of behaviors indicative of mood and anxiety disorders. These observations contribute to the body of work investigating the etiology and treatment of mood disorders for the purpose of guiding pharmaceutical drug discovery towards the eventual identification of antidepressant compounds with greater efficacy and fewer side effects.

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ROLE OF HIPPOCAMPAL NEUROGENESIS IN THE ETIOLOGY AND TREATMENT OF MOOD & ANXIETY DISORDERS

Jennifer L Onksen
A DISSERTATION
in
Pharmacology

Presented to the Faculties of the University of Pennsylvania
in
Partial Fulfillment of the Requirements for the
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ABSTRACT

ROLE OF HIPPOCAMPAL NEUROGENESIS IN THE ETIOLOGY AND TREATMENT OF MOOD & ANXIETY DISORDERS

Jennifer L Onksen
Advisor: Julie A Blendy, Ph.D

Mood disorders are associated with deficits in hippocampal size and function. Neurogenesis in the dentate gyrus of the hippocampus has been implicated in the behavioral efficacy of some antidepressants. However, the precise function of newborn hippocampal neurons in the etiology and treatment of mood disorders remains unclear. Anxiety, which is highly comorbid with mood disorders, also appears to be influenced by neurogenesis. The intricacies of this influence are poorly understood. Here, the role of neurogenesis in the pathophysiology and treatment of mood and anxiety disorders is investigated in a transgenic mouse exhibiting partial suppression of hippocampal neurogenesis. This reduction is induced by hippocampal microinjection of a Cre-expressing adeno-associated virus to delete ATR, a cell cycle checkpoint kinase, from the hippocampus of adult mice. Subsequent to hippocampal ATR deletion, behavior is examined in mouse models of anxiety, depressive-like behavior, antidepressant efficacy, and susceptibility to stress. In addition, the necessity of heightened neurogenesis in exercise-induced anxiety is examined. ATR deletion resulted in an anhedonic phenotype in a sucrose drinking task but did not increase the likelihood of developing behavioral despair in the learned helplessness paradigm. Furthermore, mice lacking hippocampal ATR exhibited reduced anxiety in a number of behavioral tests. In the novelty-induced hypophagia task, the ability of chronic antidepressants to alleviate hyponeophagia was absent in ATR-deleted mice, and this effect was linked to an attenuation of the ability of
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CHAPTER I. INTRODUCTION

A. Mood and anxiety disorders

Mood and anxiety disorders are highly prevalent mental illnesses afflicting a significant proportion of adults in the US each year. Among affected individuals, these disorders are highly variable in their etiology and treatment outcome. A better understanding of the pathophysiology of mood and anxiety disorders will not only improve disease prognosis but also ease the large financial burden these illnesses impart due to high healthcare and economic costs (Greenberg et al., 2003).

Mood disorders

Approximately 10% of adults in the US suffer from a mood disorder each year (Kessler et al., 2005). The majority suffer from dysthymic or major depressive disorder, both of which are characterized by persistent, depressive-like symptoms and differ in regards to severity. The DSM-IV further subdivides depressive disorders into numerous clinical subtypes, supporting the hypothesis that depression may represent a diverse set of pathophysiologies presenting with similar, overlapping symptoms (Akiskal, 1989; Fagiolini and Kupfer, 2003). Depression is defined as the presence of at least one two-week episode of dysphoria and/or anhedonia plus a multitude of additional symptoms including altered sleep/appetite, fatigue, agitation, inability to concentrate, and recurrent thoughts of death or suicide (American Psychiatric Association. and American Psychiatric Association. Task Force on DSM-IV., 2000). Depression is thought to be brought on by an interaction between stressful life experiences and genetic susceptibility factors (Goldberg, 2006), including alterations in the serotonin transporter gene (Caspi et al.,
Treatment for depression includes cognitive behavioral therapy, pharmacotherapy, and electroconvulsive therapy for severe, treatment resistant depression. Current pharmacotherapies act to increase levels of monoamine transmitters in the brain, either through blocking their reuptake at the synapse or through inhibiting their degradation. Because these drugs require 4-6 weeks to relieve symptoms of depression, they are thought to induce downstream changes in gene transcription and neuronal plasticity following chronic administration (Hyman and Nestler, 1996). Current pharmacotherapies often produce unwanted side effects and less than half of patients show full remission of their disease, even following trials of multiple drugs (Berton and Nestler, 2006).

Anxiety Disorders

Approximately 18% of adults in the US experience an anxiety disorder each year (Kessler et al., 2005), including general anxiety, panic disorder, social anxiety, post-traumatic stress disorder, and obsessive-compulsive disorder. Anxiety disorders are marked by pervasive worry, tension and avoidance (Allgulander, 2006) and as such, components of fear circuitry in the brain, including the amygdala, nucleus accumbens, hippocampus and insular cortex are implicated in animal models (Kent and Rauch, 2003; Davis, 2006; Maren, 2008). Treatment for anxiety includes benzodiazepines, anticonvulsants, and the serotonin and norepinephrine reuptake inhibitors commonly used in the treatment of depression (Baldwin et al., 2011). Depression and anxiety disorders are highly comorbid; up to 60% of depressed individuals also suffer from anxiety symptoms (Kessler et al., 2003). This comorbidity, in addition the ability of
selective serotonin reuptake inhibitors (SSRIs) to treat both disorders (Sramek et al., 2002; Millan, 2003) suggests they may share a common underlying pathophysiology.

B. Modeling mood disorders in rodents

Extensive efforts have been made to model depression in rodents in order to study the cellular and molecular changes which occur concurrent with the onset of depressive-like behavior and the reversal by antidepressant drugs. Because the etiology of depression is poorly understood, researchers often expose rodents to stressful conditions over a period of days or weeks. Changes that occur include anhedonia, behavioral despair, anxiety, alterations in gene transcription, dysfunctional HPA axis, and reduced hippocampal neurogenesis.

Modeling Depression

Unpredictable Chronic Mild Stress (UCMS)

UCMS involves exposing rodents to a variety of unpredictable stressors including dirty cages, wet bedding, food/water restriction, mild footshock, restraint, extreme temperature, and cage tilt (Willner, 2005). Unpredictability is essential to the development of a disease state, as predictable chronic stress has actually been shown to enhance mood, neurogenesis, and memory (Parihar et al., 2011). UCMS results in anhedonia, as measured by reduced interest in sucrose water, reduced brain stimulation reward, and reduced hippocampal neurogenesis (Willner et al., 1987; Papp et al., 1991; Moreau et al., 1993; Alonso et al., 2004). In addition to modeling some of the symptoms of depression observed in humans, UCMS-induced behavioral alterations can be reversed
by chronic treatment with antidepressants (Alonso et al., 2004; Luo et al., 2005; Jayatissa et al., 2006).

*Learned Helplessness (LH)*

The LH paradigm, first proposed by Overmier and Seligman (1967), causes behavioral despair and deficits in coping behavior reminiscent of symptoms observed in clinically depressed individuals. Rodents exposed to unavoidable, uncontrollable footshocks develop escape deficits when exposed to escapable footshocks. This behavior, as well as reduced cell proliferation which occurs as a result of acute footshock, can be reversed by chronic treatment with an antidepressant (Malberg and Duman, 2003). Interestingly, LH experiments in rats have demonstrated that reduced hippocampal neurogenesis does not influence susceptibility to helplessness (Vollmayr et al., 2003; Reif et al., 2004). Very little work has been done to examine neurogenesis in the context of learned helplessness in mice. In addition, numerous studies have suggested an individual susceptibility to helplessness among cohorts of animals (Vollmayr et al., 2003; Chourbaji et al., 2005).

*Chronic Social Stress*

Many stressors which lead to mental illness are of a social nature (Brown and Prudo, 1981). In primates and tree shrews, psychosocial stress leads to hippocampal deficits in dendritic structure and cell proliferation (Uno et al., 1989; Magarinos et al., 1996). In rodents, chronic social defeat by an aggressor induces numerous behavioral changes representative of negative mood state, including anhedonia, anxiety, behavioral despair, reduced motivation, social avoidance, and reduced hippocampal neurogenesis.
Stimulation of hippocampal neurogenesis may play an important role in recovery from social stress (Schloesser et al., 2010).

Exogenous Corticosterone

In response to stress, a major risk factor for depression (Kessler, 1997), the hypothalamic-pituitary-adrenal (HPA) axis is activated, leading to production of glucocorticoid stress hormones which activate glucocorticoid receptors (GR) in the hippocampus. This leads to inhibitory feedback and suppression of subsequent glucocorticoid production (Herman et al., 1989; Jacobson and Sapolsky, 1991; van Haarst et al., 1997). In depression, HPA axis dysfunction commonly occurs and as many as 50% of depressed individuals exhibit impaired GR negative feedback (Gibbons, 1964). In animal models of depression, elevated circulating corticosterone is often observed (Czeh et al., 2002; Harvey et al., 2003). Administering exogenous corticosterone is thought to mimic this aspect of stress and is thus used to investigate the impact of HPA axis activation on depressive-like behaviors. Chronic low dose corticosterone reduces hippocampal neurogenesis and induces depressive-like behavior in rodents (Murray et al., 2008; Zhao et al., 2008; Crupi et al., 2010).

Forced Swim Test (FST), Tail Suspension Test (TST)

In the FST, a rodent is placed into a beaker of water and the time it spends immobile is measured. In the TST, a rodent is suspended by its tail and the time immobile is measured. Immobility is interpreted as behavioral despair, a possible symptom of depression. While the construct and face validity of this model may be
called into question, immobility can be reduced by acute and chronic treatment with most currently prescribed antidepressants, suggesting strong predictive validity for identifying new potential antidepressants. Thus, these tests are often used as screens for antidepressants (Lucki, 1997; Porsolt, 2000), rather than measures of depressive-like behavior. However, it is not known whether only monoamine based drugs can be detected, or if reduced immobility generalizes to antidepressant drugs with different mechanisms of action, as compounds with non-monoamine mechanisms are not widely used to treat depression as of yet (Nestler et al., 2002).

**Modeling Anxiety**

Mouse models of anxiety can be categorized as conditioned response tests or unconditioned response tests (Belzung and Griebel, 2001, for review). Conditioned response tests involve a stressful event and include conflict tests, active/passive avoidance, and defensive burying. Unconditioned response tests are most commonly used to measure anxiety and include exploration tests (maze, holeboard, dark/light choice, open field), social tests, startle responses and separation or shock-induced ultrasonic vocalizations. Many of these tests take advantage of the natural behavior of mice to avoid bright, open, novel and/or aversive stimuli. Anxiety tests are validated by the ability of anxiolytic agents such as benzodiazepines to modify behavior. This section will cover anxiety tests relevant to the data presented in the subsequent chapters.

**Exploratory-based approach/avoidance tests**

The open field (Christmas and Maxwell, 1970), elevated zero maze (Shepherd et al., 1994), and light-dark box (Crawley and Goodwin, 1980) tests all feature an
environment in which a mouse has the option of exploring an adverse environment or remaining in a less anxiety-provoking location within the test arena. Treatment with benzodiazepines reduces anxiety as measured by increased exploration of the open arms of the zero maze, the light compartment of the light-dark box, and the center of the open field.

Marble burying

Rodents will use bedding material to bury unfamiliar objects in their cage in order to avoid what they perceive as a potential threat (Archer et al., 1987). Benzodiazepines (Broekkamp et al., 1986; Archer et al., 1987), as well as certain antidepressant compounds (Borsini et al., 2002), reduce marble burying without altering locomotor activity (Nicolas et al., 2006). It has been proposed that marble burying models components of obsessive-compulsive disorder, rather than anxiety per se (Albelda and Joel, 2011). Nonetheless, the proven efficacy of anxiolytic agents to change behavior in this test makes it a useful measure of anxiety-like behavior when used in conjunction with other tests.

Hyponeophagia

Hyponeophagia paradigms serve a dual purpose: measuring anxiety state and modeling chronic antidepressant treatment. Hyponeophagia paradigms come in two varieties, novelty-suppressed feeding (NSF), and novelty-induced hyponeophagia (NIH) (Bodnoff et al., 1988; Dulawa et al., 2004). In general, these paradigms measure latency to consume food in a novel, anxiety-provoking environment. In the NSF paradigm, mice are food-deprived and subsequently given access to a regular food
pellet in a novel environment. In the NIH paradigm, mice are trained to consume a palatable food in their home cage, and subsequently presented with the opportunity to consume the palatable food in a novel environment, in a fed state. In hyponeophagia paradigms, mice face a conflict between avoiding a novel environment and the desire to feed (Merali et al., 2003; Dulawa and Hen, 2005). Thus, this is a test of anxiety, and latency is reduced by acute administration of a benzodiazepine. Additionally, chronic treatment with antidepressants, but not acute treatment, will reduce latency to feed, suggesting this is a valuable model for evaluating the cellular and molecular mechanisms of antidepressant drugs. Of interest, ablating hippocampal neurogenesis with x-ray irradiation blocks the efficacy of two antidepressants, the SSRI fluoxetine and the tricyclic desipramine (Santarelli et al., 2003).

C. Hippocampal neurogenesis

The hippocampus is located in the medial temporal lobe of the brain, makes up part of the limbic system, and is important for consolidating information into memory and for spatial navigation. In addition, the hippocampus has been implicated in the pathophysiology of mood disorders. Clinical studies in depressed individuals have demonstrated reduced hippocampal volume which is reversed by antidepressant treatment (Sheline et al., 1999). Additionally, reduced hippocampal volume may be indicative of increased vulnerability to psychiatric illness (Gilbertson et al., 2002). The hippocampus shares a unique feature with just one other brain region, the ability to produce a substantial amount of new neurons through adulthood, a process called hippocampal neurogenesis. As discussed below, extensive research has focused on
understanding the process of hippocampal neurogenesis and the function of these newborn neurons, especially in the context of mood disorders and their treatment.

**Characterizing neurogenesis**

Hippocampal neurogenesis has been observed in rodents (Kaplan and Hinds, 1977), non-human primates (Gould et al., 1999), as well as in humans (Eriksson et al., 1998). Within the dentate gyrus of the hippocampus, multipotent progenitor cells in the subgranular zone give rise to new-born neurons which migrate to the inner granule cell layer and mature to give rise to granule neurons (For exhaustive review, see Ming and Song, 2005). These cells extend axonal projections to the CA3 pyramidal cell layer (Hastings and Gould, 1999), while their dendrites extend into the molecular layer and grow increasingly complex over a period of months (van Praag et al., 2002). Functional integration begins as soon as one week after proliferation and the newborn neurons continue to form additional synaptic connections in the subsequent months. Young neurons exhibit unique properties compared to more mature granule neurons, including a lower threshold for excitation (Schmidt-Hieber et al., 2004) and long-term potentiation (Wang et al., 2000).

Newborn neurons are essential for certain types of hippocampal-dependent learning, including pattern separation (Clelland et al., 2009; Aimone et al., 2010; Sahay et al., 2011), the ability to encode and subsequently differentiate between highly similar events or stimuli. The exact role of new neurons in emotional regulation and mood state is not so clear.

Research into hippocampal neurogenesis has identified an extensive list of intrinsic and extrinsic factors, ranging from behavioral (Lucassen et al., 2010) to
molecular (Hagg, 2005), which regulate all stages of neurogenesis. Factors which increase neurogenesis include mood-improving antidepressant compounds (Malberg et al., 2000; Manev et al., 2001; Nakagawa et al., 2002), exercise (van Praag et al., 1999), environmental enrichment (Kempermann et al., 1997; Brown et al., 2003; Llorens-Martin et al., 2007) and learning (Gould et al., 1999). Factors which contribute to reduced hippocampal neurogenesis include various forms of stress (For review, Warner-Schmidt and Duman, 2006), aging (Eckenhoff and Rakic, 1988; Kuhn et al., 1996) and disease (Danzer, 2008). In addition to the aforementioned endogenous stimuli, numerous proteins, neurotransmitters, hormones and epigenetic regulation modulate the proliferation, differentiation, or survival of newborn neurons (For review, Hagg, 2005; Hsieh and Eisch, 2010). Finally, a number of transgenic mice exist in which aspects of hippocampal neurogenesis is altered; models relevant to the work presented in the subsequent chapters will be covered below.

**Neurogenesis in the etiology of mood and anxiety disorders**

The neurogenesis hypothesis of depression was originally formulated based on extensive evidence that stress, a major risk factor for depression, suppressed hippocampal neurogenesis while pharmacotherapeutic antidepressants elevated neurogenesis. However, more recent work in rodent models indicates depressed mood can be elicited through various forms of stress without necessarily inhibiting neurogenesis. In addition, a number of non-neurogenic phenomena have been linked to the onset of, and recovery from, depressive-like behavior in rodents, including dentate gyrus evoked activity (Airan et al., 2007), hippocampal circuit dynamics (Meltzer et al., 2005), neuronal remodeling (Bessa et al., 2009) and neuroplasticity (Shors et al., 1989).
Many of these phenomena are hippocampal and may be influenced by neurogenesis, but they nonetheless support the theory that reduced neurogenesis alone does not guarantee depressed mood in all circumstances.

The relationship between neurogenesis and anxiety is currently not clear, as the outcomes of studies investigating this possible relationship are inconsistent. Exercise, which enhances neurogenesis, has been shown to both increase (Burghardt et al., 2004; Fuss et al., 2010) and decrease anxiety (Dishman et al., 1996; Salam et al., 2009), suggesting a complex relationship dependent on rodent strain, housing conditions, exercise protocol, timing of tests in relation to running, and particular tasks utilized to measure anxiety. At the other end of the spectrum, mouse models of suppressed hippocampal neurogenesis do no better in clarifying the neurogenesis/anxiety relationship. Some studies observe no link (Saxe et al., 2006), while others show heightened (Bergami et al., 2009; Revest et al., 2009) or reduced (Uchida et al., 2011) anxiety in conjunction with suppressed neurogenesis.

Thus, evaluating depressive and anxiety-like behaviors in the mouse model of reduced neurogenesis employed in the subsequent chapters was an important aim of this dissertation.

**Neurogenesis in the treatment of mood and anxiety disorders**

In contrast to the dearth of evidence supporting reduced neurogenesis as a causal factor in the onset of depression, enhanced hippocampal neurogenesis may be essential to the efficacy of specific antidepressant pharmacotherapies in certain behavioral measures. Chronic administration of antidepressants with monoaminergic mechanisms require hippocampal neurogenesis for their efficacy in a hyponeophagia
task (Santarelli et al., 2003) and for reversal of the behavioral effects of chronic mild stress (Surget et al., 2008). Antidepressant drugs with alternative mechanisms of action, including an MCHR1 antagonist, CRF1 antagonist and vasopressin 1b antagonist, are effective in relieving depression and anxiety-like behaviors in mice x-ray irradiated to block neurogenesis (David et al., 2007; Surget et al., 2008). More recently, in a chronic corticosterone model of anxiety/depressive like state, fluoxetine was found to require neurogenesis for mood improving actions in some, but not all behavioral tests, suggesting particular drugs can exert both neurogenesis-dependent and neurogenesis-independent actions (David et al., 2009).

Furthermore, not all mood-improving stimuli require neurogenesis. Environmental enrichment, which is shown to increase hippocampal neurogenesis, alleviates hyponeophagia and reduces anxiety in mice x-ray irradiated to block neurogenesis (Meshi et al., 2006). The requirement of neurogenesis for antidepressant efficacy is also strain dependent, as demonstrated in BALB/cJ mice (Holick et al., 2008).

D. Methods of manipulating neurogenesis in rodents

With the goal of identifying the functional relevance of neurogenesis and the behaviors influenced by newborn neurons, a number of methods have been developed to suppress neurogenesis including chemical treatment, irradiation, and genetic manipulation.

Chemical treatment

Methylazoxymethanol acetate (MAM) is a toxin which inhibits proliferating cells. It was used in adult rats to demonstrate a role of newborn neurons in certain types of learning and memory (Shors et al., 2002; Bruel-Jungerman et al., 2005). MAM was later
shown to alter health at the dose necessary to reduce neurogenesis and is utilized infrequently today (Dupret et al., 2005).

**X-ray irradiation**

A popular approach to studying the function of neural stem/progenitor cells in the hippocampus is to expose the brain region to focal x-ray irradiation. A single 15-gray exposure results in at least 85% suppression of neurogenesis and lasts for up to 3 months (McGinn et al., 2008). This is a consistent, reproducible approach that allows for the generation of a large cohort of neurogenesis-deficient mice in a short time. However, there are some drawbacks to the x-ray irradiation approach. X-ray irradiation can affect differentiation, increase activated microglia and disrupt angiogenesis (Tada et al., 2000; Monje et al., 2002; Mizumatsu et al., 2003). Nonetheless, this method has been essential in advancing the field’s understanding of the role of new neurons, including in the first study to demonstrate a causal link between neurogenesis and the efficacy of antidepressant drugs (Santarelli et al., 2003).

**Genetic models**

To compliment and confirm the findings of x-ray irradiation studies, researchers have developed transgenic approaches to ablating neurogenesis. The first to be used directly alongside x-ray irradiation was a transgenic mouse in which herpes simplex virus thymidine kinase (TK) is expressed under control of the GFAP promoter (Saxe et al., 2006; Saxe et al., 2007). Proliferating cells in the hippocampus are GFAP-expressing. Subsequent administration of ganciclovir (GCV) kills proliferating, TK-positive cells, thus ablating neurogenesis. To reduce toxicity and enhance specificity (Bush et al., 1998;
Bush et al., 1999), this model was later expanded to the nestin promoter, such that nestin-expressing progenitor cells would be killed upon administration of ganciclovir, with similar effects on neurogenesis (Singer et al., 2009). Another similar approach involves doxycycline-inducible over-expression of the pro-apoptotic factor Bax in neural progenitor cells through a Nestin promoter (Revest et al., 2009). This results in a 50% reduction in neurogenesis and altered mood/anxiety-related behaviors.

A number of proteins important for cell cycle regulation, as well as those upstream of direct cell cycle regulation, are necessary for normal hippocampal neurogenesis, including Cdk5, Bmi1 and cyclin D2 (Kowalczyk et al., 2004; Zencak et al., 2005; Lagace et al., 2008; Jaholkowski et al., 2009). Deletion of these various cell cycle elements results in either partial or complete attenuation of neurogenesis, generating models for investigating the functional relevance of neurogenesis. ATR, as a cell cycle checkpoint kinase, falls into this category.

As previously mentioned, an extensive list of extrinsic and intrinsic factors regulate hippocampal neurogenesis. This is exemplified by the many transgenic mice which exhibit altered hippocampal neurogenesis and alterations in mood and anxiety-like behaviors. Genes, and their protein products, shown to influence hippocampal neurogenesis include p21Cip1, GABA_A γ2 subunit, p75 neurotrophin receptor, AMPA receptor GluA1 subunit, p11 (S100A10), heat shock factor 1 (HSF1), Kruppel-like factor 9 (Klf9), connexin, NeuroD1, Fmr1, and macrophage migration inhibitory factor (MMIF) (Earnheart et al., 2007; Catts et al., 2008; Pechnick et al., 2008; Eadie et al., 2009; Gao et al., 2009; Kunze et al., 2009; Egeland et al., 2010; Conboy et al., 2011; Procaccini et al., 2011). These transgenic models present numerous caveats including developmental
effects and non-specific effects. Nonetheless, they provide additional clues and support for a complex relationship between neurogenesis and behavior.

**E. Crelox-conditional ATR knockout**

**ATR (Ataxia-telangiectasia mutated (ATM) and Rad3-related)**

ATR is a cell cycle checkpoint kinase and an essential regulator of DNA replication. It is necessary for stabilization of the replication fork during a stall in DNA replication. In its absence, double strand breaks and subsequent loss of genomic integrity are observed (Paulsen and Cimprich, 2007). In cell culture, cell cycle arrest and cell death occur in murine embryonic fibroblasts and human cells lacking ATR (Cortez et al., 2001; Brown and Baltimore, 2003). In mouse embryos, constitutive deletion of ATR leads to chromosomal fragmentation and early embryonic lethality; cultured blastocysts die prior to embryonic day 7 by caspase-independent apoptosis, implying loss of genomic integrity (Brown and Baltimore, 2000).

**ATR deletion from the adult mouse**

Ubiquitous deletion of ATR from the adult mouse leads to aging-like phenotypes associated with premature depletion of stem and progenitor cell pools (Ruzankina et al., 2007). This observation made ATR a strong potential candidate for suppressing neurogenesis in the hippocampus, as it depends on a pool of progenitor cells in the subgranular zone. Ruzankina and colleagues also demonstrated that ATR could be deleted from mature forebrain CNS neurons, including those in the hippocampus, by *Synapsin1* promoter-driven Cre expression, with no effects on circadian period,
coordination, strength, anxiety, or hippocampal dependent learning. These observations suggest ATR is not necessary for the function of mature neurons in the hippocampus and implicates loss of ATR from the proliferating cell population in the behavioral consequences that we observe following hippocampal-specific deletion.

**Targeting ATR in the hippocampus**

Hippocampal ATR deletion was achieved by microinjecting an adeno-associated virus (AAV) encoding Cre recombinase (Cre) directly into the hippocampus of mice with loxP sequences flanking the kinase domain of ATR (ATR^ff). An AAV was selected due to its persistent expression and minimal evoked immune response (Cearley and Wolfe, 2006). AAV expression peaked at approximately 4 weeks and all experiments were conducted at least 6 weeks following AAV injection to assure deletion of ATR and suppression of neurogenesis. In addition, we treated ATR^ff mice carrying a tamoxifen-inducible Cre transgene (ATR^ff;CreERT2) with tamoxifen to induce a ubiquitous ATR deletion. This was done to verify that the cellular and behavioral effects of the AAV injection were due to ATR deletion, and not an undesired side effect of the AAV. In the subsequent chapters, ATR deletion is utilized as a model of suppressed hippocampal neurogenesis to explore the influence of neurogenesis in the onset and treatment of mood and anxiety disorders, as well as to examine the neurogenesis-dependent effects of exercise, which is a potent stimulator of neurogenesis and a mood-improving stimulus in the human population.
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CHAPTER II

Selective Deletion of a Cell Cycle Checkpoint Kinase (ATR) Reduces Neurogenesis and Alters Responses in Rodent Models of Behavioral Affect

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Abstract

Hippocampal function has been implicated in mood and anxiety disorders, as well as in the response to antidepressant (AD) treatment. However, the significance of new neurons in the therapeutic mechanism of ADs remains unclear. In this study the proliferation of new neurons was inhibited through conditional deletion of ataxia telangiectasia-mutated and rad-3 related (ATR), a cell cycle checkpoint kinase, and cellular and behavioral outcomes following AD exposure were evaluated. ATR was conditionally deleted by microinjecting a Cre recombinase-expressing virus into the hippocampus of floxed-ATR mice. Behavioral assessment in multiple rodent models of affective state revealed anxiolytic-like behavior in the elevated zero maze, marble burying test, and novelty-induced hypophagia (NIH) test. The efficacy of chronic desipramine (DMI) treatment was evaluated in the NIH test, as this paradigm is thought to be sensitive to increases in neurogenesis by chronic AD exposure. Chronic exposure to DMI reduced hyponeophagia in the NIH test in control mice, whereas DMI had no behavioral effect in ATR-deleted mice. While DMI did not alter cell proliferation in either group, it did produce a robust increase in dendritic spine density in control mice, indicative of enhanced neuronal plasticity. This effect of DMI on spine density was severely attenuated following ATR deletion. These findings demonstrate that reductions in basal neurogenesis produce an anxiolytic phenotype and reduce AD efficacy in behaviors requiring chronic exposure. Attenuated capacity for synaptic remodeling may underlie this reduced AD efficacy. ATR deletion may serve as a valuable model to study the various proposed roles of newborn neurons in the hippocampus.
Introduction

Major depression is a highly prevalent psychiatric disorder characterized by a diverse manifestation and etiology. Depression and anxiety are highly co-morbid and pervasive (Merikangas et al., 2003). The ability of chronic antidepressant (AD) treatment to ameliorate the symptoms of both depression and anxiety (Schoevers et al., 2008) raises the possibility that they may share common molecular underpinnings. Hippocampal neurogenesis has been implicated in the therapeutic efficacy of AD treatment. This theory is supported by evidence that the maturation and functional integration of new neurons occurs over a period of time similar to that required for ADs to exert their therapeutic effects (Malberg et al., 2000; Jessberger and Kempermann, 2003). However, the significance of new hippocampal neurons for the onset of depressive-like symptoms remains unclear (Balu and Lucki, 2009, for review). While rodent models of chronic stress can reduce neurogenesis (Malberg and Duman, 2003; Murray et al., 2008; Elizalde et al., 2010) and induce anhedonia (Papp et al., 1991), reduced neurogenesis alone is not sufficient to induce depression and is more likely a risk factor contributing to the onset of depressive-like symptoms (Sahay and Hen, 2007).

Despite early promising reports, the relevance of new neurons to antidepressant effects is still unclear. Fluoxetine (FLX) was shown to be ineffective in a behavioral model of AD efficacy following ablation of hippocampal neurogenesis by x-ray irradiation (Santarelli et al., 2003). Additionally, in a comparison of the inbred mouse strains MRL/MjP and C57BL/6, Balu and colleagues found behavioral efficacy following FLX and desipramine (DMI) treatment only in the strain that exhibited a positive neurogenic response to these drugs (Balu et al., 2009). In contrast, there is evidence that AD
treatment elicits behavioral changes independent of hippocampal neurogenesis (David et al., 2007; Holick et al., 2008; Huang et al., 2008). Furthermore, numerous studies implicate enhanced neuroplasticity and synaptic function in the therapeutic efficacy of ADs (Bessa et al., 2009; Calabrese et al., 2009, for review; O'Leary et al., 2009; Marchetti et al., 2010).

Although depression and anxiety present with high co-morbidity, little work has been done to examine neurogenesis in the context of anxiety. Some studies find no link (Saxe et al., 2006), while others observe an anxiogenic response associated with reduced neurogenesis (Bergami et al., 2009; Revest et al., 2009). Voluntary exercise leads to a robust increase in neurogenesis while also increasing anxiety-like behavior (Fuss et al., 2010). However, it must also be noted that voluntary exercise can reduce anxiety (Duman et al., 2008; Salam et al., 2009), suggesting a variety of factors in addition to neurogenesis likely interact to modulate anxiety.

To address the relevance of neurogenesis to psychiatric illness, we have utilized a genetic approach in which hippocampal neurogenesis is reduced following deletion of ATR. ATR is necessary for genomic stability in the early embryo and cells lacking ATR ultimately exit the cell cycle or undergo programmed cell death (Brown and Baltimore, 2000; Brown and Baltimore, 2003). Ubiquitous deletion of ATR from the adult mouse leads to age-related phenotypes that are associated with the premature depletion of stem/progenitor cells (Ruzankina et al., 2007). Previous research has demonstrated that ATR can be deleted from differentiated forebrain CNS neurons by Synapsin1 promoter-driven Cre expression with no effects on circadian period, coordination, strength, anxiety, or hippocampal-dependent learning (Ruzankina et al., 2007). This suggests that ATR is not necessary for the function of differentiated neurons and implicates loss of
ATR from the proliferating cell population in the behavioral consequences that we observe following hippocampal-specific deletion. Targeting a protein that is not necessary for the function of mature cells provides the added benefit of minimizing possible compensatory or non-specific effects that could arise if the deleted protein were to play a role outside of cell division.

Here, we show that deletion of ATR from the adult hippocampus leads to at least a 50% reduction in neurogenesis. Behavioral consequences of partially inhibiting neurogenesis have been observed previously (Revest et al., 2009; Conboy et al., 2010). We thus evaluated behavior in rodent models of affective state and examined the cellular and behavioral effects of AD treatment following ATR deletion. While not a complete ablation, this model has the potential to mimic the reduced rate of neurogenesis that is observed during aging (Bondolfi et al., 2004). Aged mice have a reduced capacity for AD-induced neurogenesis (Couillard-Despres et al., 2009) and treatment-resistant depression is prevalent in the aged population (Lenze et al., 2008).

**Materials and Methods**

**Animals** Mice homozygous for the Cre/lox-conditional allele of ATR (referred to as floxed-ATR or ATR<sup>f/f</sup>) and mice expressing the tamoxifen-inducible Cre transgene (CreERT<sub>2</sub>) on a 129SV/C57BL/6 background were generated as previously described (Ruzankina et al., 2007). Floxed-ATR mice were used for hippocampal-specific ATR deletion. For whole-body deletion of ATR, floxed-ATR mice carrying CreERT<sub>2</sub> were treated with tamoxifen as described (Ruzankina et al., 2007). For behavioral studies in these mice, one of two control groups was utilized: (1) ATR<sup>f/f</sup>;CreERT<sub>2</sub> + vehicle, or (2)
ATR<sup>ff</sup> + tamoxifen. Mice (1.5-5 months old, 20-35 gm) were housed in groups of 3-4 following stereotaxic surgery and during experimental paradigms, with exceptions noted. All behavioral experiments were conducted on males and females at least 5 weeks following stereotaxic surgery or tamoxifen treatment. Animals were maintained on a 12 hr light/dark cycle with food and water available ad libitum in accordance with the University of Pennsylvania Institutional Animal Care and Use Committee. In some instances multiple behavioral tests were performed in a single cohort of mice, as indicated here: (1) Elevated zero maze was performed 3 days following marble burying; (2) Training in the NIH commenced 3 days following forced swim test; (3) Locomotor activity was measured 2 days prior to the Morris water maze and restraint stress was measured 1 week following water maze testing.

**Adeno-associated virus** Adeno-associated viruses (AAV) expressing Cre recombinase (AAV2/9.CMV.PI.CRE, titer $2.84 \times 10^{13}$ gc/ml) and eGFP (AAV2/9.CMV.eGFP, titer $3.74 \times 10^{13}$ gc/ml) were generated by the University of Pennsylvania Vector Core. The expression cassette consists of the AAV2 terminal repeats flanking the CMV promoter-PI-Cre recombinase/eGFP sequences packaged into AAV9. The vectors were purified using CsCl sedimentation method. Quantification of vector genome copies (gc) was performed by Q-PCR. AAVs were diluted in sterile PBS for microinjections.

**Hippocampal Injections** Floxed-ATR mice (6-8 weeks) were anesthetized with isoflurane and secured in a stereotaxic frame (Kopf, Tujuna, CA). Holes were drilled bilaterally in the skull at sites of injection. Stereotaxic coordinates for the hippocampus are (from Bregma) anterior-posterior -2.1, lateral +/- 1.4, dorso-ventral -2.0, and
anterior-posterior -2.9, lateral +/− 3.0, dorso-ventral -3.8. 0.5 ul of \(1 \times 10^{10}\) gc/ul AAV was injected at each site through a 33 gauge needle on a 5 ul Hamilton syringe using a KDS310 Nano Pump (KD Scientific, Holliston, MA) mounted to the stereotaxic frame, at a rate of 0.1 ul/min. The needle remained in place for 4 additional minutes at each injection site. The skin was sutured and the animal recovered on a heating pad before returning to the home cage.

**Drugs** Desipramine (DMI, Sigma, St. Louis, MO), chlordiazepoxide (CDP, Sigma) and bromodeoxyuridine (BrdU, Sigma) were dissolved in 0.9% saline. Solutions were prepared immediately before use and injected intraperitoneally using a volume of 10 ml/kg. For cell proliferation analysis, 200 mg/kg BrdU was administered 24 hours prior to perfusion. For cell survival analysis, 100 mg/kg BrdU was administered once per day for 4 days, 4 weeks prior to perfusion. To activate the inducible CreERT\(_2\), tamoxifen (Sigma) was dissolved at a concentration of 20 mg/ml in a mixture of 98% corn oil / 2% ethanol and was injected intraperitoneally once per day for 4 days.

**Quantitative-PCR** Cre/Lox recombination was quantified using RT-PCR. Brains were rapidly removed and hippocampi were dissected out and flash-frozen in liquid nitrogen. DNA was extracted using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Deletion rate was analyzed by utilizing a Taqman primer/probe set which overlap the 3’ lox sequence and a portion of the excised domain as described (Ruzankina et al., 2009). Floxed-ATR levels were normalized to the housekeeping gene TBP.
**Immunohistochemistry**  Mice were anesthetized with nembutol (10 mg/kg) and transcardially perfused with cold 0.1 M phosphate-buffered saline (PBS) for 5 minutes, followed by cold 4% paraformaldehyde (PFA) in PBS for 15 minutes. Brains were postfixed overnight in PFA at 4°C and subsequently stored at 4°C in 30% sucrose. Brains were frozen on dry ice, sectioned coronally at a thickness of 40 µm, and transferred to PBS + 0.5% Sodium Azide at 4°C prior to processing for immunohistochemistry.

For BrdU analysis, sections were wet-mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA) for stereological analysis using a modified version of the optical fractionator method (West et al., 1991). Mounted sections were incubated in 0.1 M citric acid, pH 6.0, for antigen retrieval. Subsequently, slides were incubated in trypsin, 2N HCl, then primary antibody (mouse anti-BrdU, 1:200, Becton Dickinson) with 0.5% Tween 20 overnight. On the second day, slides were incubated for 1 hour in secondary antibody (biotinylated anti-mouse IgG, Vector Laboratories), 1 hour in avidin-biotin-HRP (1:100, Vector Laboratories), and labeled cells were visualized using diaminobenzidine (Sigma). PBS-washed slides were then counterstained and coverslipped with Permount. BrdU-IR cells in every 9th section of the hippocampus were counted with a 100x oil immersion lens, omitting cells in the upper-most focal plane. Cells within clusters were counted by distinguishing nuclear borders while focusing down through the tissue using an objective with a narrow depth of focus. For proliferation counts, a cell is included in the subgranular zone (SGZ) of the dentate gyrus if it is touching, or within two cells of the granule cell layer (GCL).

For doublecortin (DCX) analysis, free-floating sections of a 1/9 series through the hippocampus were blocked for 1 hour with 3% Normal Horse Serum, 0.5% Tween 20 and 0.2% Triton in PBS (blocking solution). Sections were then incubated for 72 hours
at 4°C in primary antibody (Goat anti-DCX, Santa Cruz #8066) diluted 1:500 in blocking solution. Sections were washed and incubated for 1 hour in secondary antibody (Horse anti-goat, Vector Laboratories) diluted 1:200 in blocking solution. Sections were then washed and treated with 0.75% H₂O₂ for 20 minutes prior to incubation in avidin-biotin-HRP (1:200, Vector Laboratories). Labeled cells were visualized using nickel-enhanced diaminobenzidine. Free-floating sections were mounted onto slides and dried overnight before dehydrating and cover-slipping with Permount. DCX-IR cells in every 9th section were counted with a 100x oil immersion lens, omitting cells in the upper-most focal plane. All cells located within the GCL and SGZ were counted.

**Golgi Impregnation**  Golgi Impregnation was conducted to label neurons and their processes for analysis of dendritic spine density according to the FD Rapid GolgiStain Kit (FD NeuroTechnologies, Ellicott City, MD). 100 µm sections were cleared with xylenes and cover-slipped with Permount mounting medium. Hippocampal CA1 neurons selected for analysis had the following characteristics: Dark and complete impregnation, untruncated tertiary dendrites, and isolated cell body with a clear connection to dendrites that are isolated from neighboring cells to avoid interference. For each brain, five CA1 neurons were selected for analysis. Within each neuron, six separate 10 µm segments of tertiary dendrite, located at least 50 µm from the cell body, were selected for counting. Number of spines per 10 µm was determined manually using a confocal microscope (Nikon Eclipse E600, Nikon) with a 100x oil immersion lens. For each treatment group, average spine density of each neuron analyzed was averaged to arrive at the spine density used for statistical analysis.
**Elevated Zero Maze**  The elevated zero maze test was performed as described previously (Gundersen and Blendy, 2009). The maze (Stoelting, Wood Dale, IL) consists of two open areas (wall height, 0.5”) and two closed areas (wall height, 12”), and is elevated 24” from the ground. Lighting in the maze is 15 lux. At the start of testing, mice were placed into one of the closed areas and were allowed to explore the maze for 300 seconds. The Viewpoint Tracking System (Viewpoint, Champagne au Mont d’or, France) was used to track time spent in the open areas, number of entries into the open areas, and distance traveled in each area.

**Marble Burying**  After a 1-hour acclimation period in the testing room, mice were placed in a standard mouse cage containing 5 cm of bedding and 20 marbles, spaced equally along the edge of the cage. A wire-mesh lid was placed on the cage. Number of marbles buried at the end of a 15-minute session was quantified. A marble was considered buried if it was at least 2/3 beneath the surface of the bedding.

**Novelty-Induced Hypophagia (NIH) Test**  The NIH test was performed as previously described (Gur et al., 2007). Briefly, mice were pair-housed and trained to consume peanut butter chips (Nestle) in their home cage. Drug treatment occurred between the training and testing periods. For chronic AD treatment, mice received 25 days of desipramine (12.5 mg/kg DMI, b.i.d.). This was followed by measuring latency to consume the palatable food on two consecutive test days. The first test day took place in the home cage. On the second test day, latency was measured in a novel environment, which consisted of an empty cage with no bedding, placed in a white box with a bright light (2150 lux) and a novel smell (dilute pine sol). On home and novel test
days, mice received their first daily injection of DMI 1 hour prior to testing latency to feed, and received their second injection of DMI in the evening, consistent with the chronic treatment paradigm. For acute CDP treatment, home and novel test days occur immediately following the training period. CDP (10 mg/kg) is injected 30 minutes prior to the novel test.

**Forced Swim Test** Mice were placed in water (15 cm depth, 23 C) in plastic cylinders (23 cm tall x 14 cm diameter) for 6 minutes. Using Viewpoint automated scoring (Viewpoint, Champagne au Mont d’Or, France), duration of immobility was measured. Mice were exposed to the swim tanks twice (pre-swim and swim test), with 24 hours between exposures. Three injections of DMI were given during the inter-swim interval: One 24 hours prior to the swim test (10 mg/kg), a second injection 5 hours prior to the swim test (10 mg/kg), and a third injection 1 hour prior to the swim test (20 mg/kg).

**Locomotor Activity** Locomotor activity was measured by beam-breaks in a photobeam frame (Med Associates, St. Albans, VT, USA). During the test, mice were placed individually into a clean home cage resting within the photobeam frame, and data were recorded by Med Associates Software. Ambulations and crossings were measured in 5-min bins for 60 minutes.

**Restraint Stress** Mice were placed in 50 mL Falcon conical vials for 15 minutes. Holes were drilled at both ends to allow for breathing and to give access to the tail. Tail blood (20 uL) was collected at 0, 15, 30, and 90 minutes. Blood was transferred directly into tubes with chilled EDTA, centrifuged at 5,000 RPM for 10 minutes at 4 C, and serum was
used to measure corticosterone levels with a $^{125}$I Corticosterone RIA kit (MP Biomedicals, Orangeburg, NY).

**Fear Conditioning** Fear conditioning was performed in a rectangular chamber capable of delivering footshocks (Med Associates, St. Albans, VT). During the training session, mice were exposed to a 20 second, 2000 Hz tone (conditioned stimulus, CS) at 2 and 3.5 minutes. During the final 2 seconds of each CS, a 0.5 mA scrambled footshock (unconditioned stimulus, US) was delivered. The training session lasted for a total of 6 minutes, at which time the mice were returned to their home cages. To evaluate contextual fear learning, 24 hours after training the mice were placed in the chamber for 6 minutes in the absence of the CS or US and their freezing behavior was assessed. To evaluate cued fear conditioning, 24 hours after the contextual fear test the mice were placed in an altered chamber and exposed to the CS at 2 minutes. They were removed from the chamber after a total of 5 minutes and freezing behavior was assessed before and after the presentation of the CS. To alter the context of the chamber for the cued fear test, an insert was placed in the chamber to modify its shape and color. Additionally, a novel scent and increased lighting were present.

**Morris Water Maze (MWM)** *MWM Apparatus:* The water maze apparatus consisted of a white circular swimming pool 150 cm in diameter, located in the center of a square-shaped room with unique spatial cues on each wall. The water was made opaque by the addition of a non-toxic white tempera paint and maintained at 23 C. The escape platform consisted of a translucent square plastic platform 12 cm in length. The water level was maintained at depth such that the surface of the hidden platform was 1 cm
below the water surface. A video camera fixed to the ceiling above the pool recorded behavior. For swim path analysis the videos were replayed on a computer equipped with Viewpoint video tracking system. The tracking system allowed for automated determination of latency to the escape platform, time spent in each quadrant, and total path length (distance traveled).

*MWM Training:* Mice were trained during the light cycle with three trials per day and a 10-minute inter-trial interval. Mice that failed to locate the hidden platform after 60 seconds were guided to the platform. Upon reaching the platform, mice remained in place for 10 seconds prior to being returned to the home cage. For hidden platform acquisition training the start location varied for each trial and was counterbalanced across the treatment groups and days. For reversal training the hidden platform was moved to a novel quadrant and the start location was held constant for each mouse. A probe test was administered 24 hours following the 9\textsuperscript{th} day of hidden platform acquisition training. For this probe test the platform was removed and mice were placed in the maze for 60 seconds and required to locate the platform using spatial extra-maze cues. Time spent in each quadrant was analyzed. 24 hours after the first probe test, reversal training commenced. Following 6 days of reversal training, a second probe-test was administered. In this 60 second reversal training probe test, time to reach the platform from a novel start location was measured. Analysis of the hidden platform training and reversal training consisted of averaging the three daily trials and comparing AAV treatments by RMANOVA.

**Statistical Analysis**  Statistical analyses were performed with GraphPad Prism 5. Data are reported as mean ± SEM. Studies comparing AAV injection were analyzed with
Student’s $t$ tests. Studies comparing AAV and environment or treatment were analyzed by two-way ANOVA followed by pair-wise comparisons with Bonferroni post hoc tests.

**Results**

**Hippocampal ATR deletion reduces neurogenesis**

To delete ATR we micro-injected AAV.Cre into both the dorsal and ventral hippocampus. AAV.Cre and the control virus, AAV.eGFP, are packaged into viral capsids of the serotype 9. AAV9 was chosen for its ability to spread throughout the hippocampus, thereby covering the entire rostral-caudal extent of the dentate gyrus without spreading outside of the hippocampal formation. Maximal deletion of ATR (-78%, $t_{16}=4.75$, $p<0.001$) was evident 8 weeks following AAV injection (Fig. 1a). Photomicrographs of eGFP fluorescence at this time point illustrate spread and expression of the AAV (Fig. 1b,c)

To determine the effect of hippocampal ATR deletion on neurogenesis, floxed-ATR mice were injected intraperitoneally (IP) with 200 mg/kg BrdU 8 weeks following hippocampal AAV microinjection and perfused 24 hours later. Compared to controls, ATR-deleted mice showed significantly reduced (-45%, $t_{17}=5.56$, $p<0.0001$) BrdU incorporation, indicating a robust reduction in basal levels of cell proliferation (Fig. 2a,d,e). To determine effect of ATR deletion on cell survival, BrdU was injected 4 weeks following AAV microinjection and mice were perfused 4 weeks later. Compared to controls, ATR-deleted mice showed significantly reduced (-57%, $t_{22}=5.31$, $p<0.0001$) BrdU incorporation, indicative of reduced cell survival (Fig. 2b) Additionally, ATR-deleted mice showed significantly reduced (-54%, $t_{17}=3.246$, $p<0.005$) expression of DCX, indicating a reduction in the total number of newly born, immature neurons present in
the hippocampus (Fig. 2c,f,g). Taken together, these data demonstrate that hippocampal ATR deletion results in at least a 50% reduction in neurogenesis. The requirement of ATR for normal rates of neurogenesis was verified in a second model of ATR deletion, ATR^{f/f};CreERT2 mice treated with tamoxifen. 5 weeks after tamoxifen treatment we already observed a 30% reduction in BrdU incorporation in these mice (p=0.06, data not shown).

**ATR deletion reduces anxiety-like behavior**

After establishing that hippocampal ATR deletion results in reduced basal levels of neurogenesis, we sought to evaluate behavior of the floxed-ATR mice in rodent models of affective state. Anxiety-like behavior was evaluated using the EZM (Fig. 3a). ATR-deleted mice spent significantly greater time ($t_{(10)}=3.26$, $p<0.01$) in the open arms of the maze, indicating reduced levels of anxiety compared to controls. This observation was not due to any overall difference in distance traveled in the maze ($t_{(10)}=0.71$, $p=0.49$) (Fig. 3b). Furthermore, deletion of ATR from the hippocampus did not affect general locomotor activity or corticosterone response to an acute stressor (Fig. S1).

The anxiolytic phenotype observed in the EZM is at odds with work by others demonstrating an anxiogenic phenotype following reduced neurogenesis (Bergami et al., 2009; Revest et al., 2009). To determine if reduced anxiety-like behavior was present in other tests, we examined behavior in the marble burying test in both the AAV and tamoxifen-inducible models of ATR deletion. As illustrated in Figure 4a, hippocampal ATR deletion was associated with a significant reduction ($t_{(28)}=3.55$, $p<0.01$) in the number of marbles buried during a 15 minute session. Consistent with the anxiolytic effect of hippocampal-specific ATR deletion, reduced anxiety-like behavior was also
observed in the marble burying test in ATR\textsuperscript{ff};CreERT\textsubscript{2} mice treated with tamoxifen ($t_{(10)}=5.805$, p<0.05) (Fig. 4b).

ATR deletion resulted in reduced hyponeophagia in the NIH test, indicative of a blunted anxiety-like response to a novel, anxiety-provoking environment. Mice were trained to consume peanut butter chips in their home cage and subsequently tested for latency to consume this palatable food in a novel, anxiety provoking environment. Following hippocampal-specific deletion, ATR-deleted mice showed significantly shorter latency to feed in the novel environment compared to controls (p<0.001) (Fig. 4c). Two way ANOVA revealed significant main effects of environment ($F_{(1,28)}=57.87$, p<0.0001) and AAV ($F_{(1,28)}=15.23$, p<0.001), as well as a significant environment x AAV interaction ($F_{(1,28)}=15.65$, p<0.001). Consistent with these findings, reduced anxiety was also observed in the ATR\textsuperscript{ff};CreERT\textsubscript{2} mice in the NIH test (Fig. 4d). Two way ANOVA revealed significant main effects of environment ($F_{(1,45)}=32.44$, p<0.0001) and genotype ($F_{(1,45)}=10.61$, p<0.005), as well as a significant environment x genotype interaction ($F_{(1,45)}=10.08$; p<0.005). *Post hoc* analyses confirmed significantly reduced latency to feed (p<0.001) in the novel environment in ATR-deleted mice compared to controls (Fig. 4d).

**Behavioral effects of DMI are attenuated following ATR deletion**

As hippocampal neurogenesis has been implicated in the mechanism of action of ADs, we next sought to determine whether ATR deletion would alter the cellular and behavioral response to chronic AD treatment. To address this we again utilized the NIH test but evaluated mice following chronic DMI treatment (12.5 mg/kg i.p., b.i.d.). This dose effectively reduces feeding latency in mice on a 129SV/C57BL/6 background.
following chronic, but not acute treatment (Gur et al., 2007; Turner et al., 2010). All training occurred prior to drug exposure. Home cage testing occurred on the 24th day of DMI treatment and novel cage testing occurred on the 25th day. Following the novel cage test, mice were given 200 mg/kg BrdU and perfused 24 hours later. There were no effects of AAV or AD (AAV main effect: $F_{(1,28)}=2.735$, $p=0.109$; AD main effect: $F_{(1,28)}=0.02$, $p=0.889$; AAV x AD interaction: $F_{(1,28)}=0.003$, $p=0.957$) on latency to feed in the home cage test (Fig. 5a). Consistent with our finding that ATR deletion reduced hyponeophagia in the absence of drug treatment (Fig. 4c), two way ANOVA revealed a significant main effect of AAV ($F_{(1,25)}=23.40$, $p<0.0001$) in the novel environment (Fig. 5a). Additionally, there was a significant main effect of DMI ($F_{(1,25)}=4.394$, $p<0.05$) and a significant AAV x AD interaction ($F_{(1,25)}=4.601$, $p<0.05$) in the novel environment (Fig. 5a). Post hoc analyses revealed a significant effect of DMI only in the AAV.eGFP treatment group ($p<0.05$), indicating that DMI does not reduce hyponeophagia in ATR-deleted mice. This is unlikely due to a floor effect of this test, as acute treatment with CDP (10 mg/kg) 30 minutes prior to the novel cage test resulted in significantly reduced latency to feed in the novel environment (Fig. 5b) in both control and ATR-deleted mice (AAV main effect: $F_{(1,24)}=4.863$, $p<0.05$; CDP main effect: $F_{(1,24)}=25.45$, $p<0.0001$). Additionally, ATR-deleted mice and controls respond equally well to DMI in the forced swim test (Fig. 5c) by demonstrating reduced immobility compared to saline-treated mice (DMI main effect: $F_{(1,32)}=30.44$, $p<0.0001$). Furthermore, the altered behavior observed in the NIH is not likely due to learning or memory impairment, as ATR deletion does not induce deficits in associative (Fig. S2) or spatial learning (Fig. S3).

**Cellular effects of chronic DMI are altered in ATR-deleted mice**
Following behavioral testing in the NIH paradigm, the cellular effects of chronic DMI treatment were evaluated. Two-way ANOVA revealed a significant main effect of AAV ($F_{(1,29)}=31.73; p<0.0001$) to reduce cell proliferation in ATR-deleted mice (Fig. 5d), as observed previously. DMI had no effect on cell proliferation in control or ATR-deleted mice (AD main effect: $F_{(1,29)}=0.173, p=0.68$, AAV x AD interaction: $F_{(1,29)}=0.877, p=0.357$). In the absence of a neurogenic effect of DMI treatment, we sought to identify a potential mechanism through which DMI may exert behavioral changes. Dendritic spine density, a correlate of synaptic transmission strength and neuronal plasticity (von Bohlen Und Halbach, 2009), can be increased by AD treatment in rodents (Norrholm and Ouimet, 2001; Hajszan et al., 2005). Reduced spine density has been observed in post-mortem tissue of depressed individuals (Law et al., 2004). To this end, we examined whether DMI exerted differential effects on CA1 dendritic spine density in ATR-deleted mice. Two-way ANOVA revealed significant main effects of AAV ($F_{(1,145)}=25.2, p<0.0001$), AD ($F_{(1,145)}=159.7, p<0.0001$), and a significant AAV x AD interaction ($F_{(1,145)}=81.41, p<0.0001$). Post hoc analyses confirmed a robust significant effect (+34%, $p<0.001$) of DMI in control mice (Fig 6a,b). This effect was greatly attenuated (+5%, $p<0.05$) in ATR-deleted mice (Fig. 6c).
Figure 1. Hippocampal microinjection of AAV.Cre deletes ATR.

A Floxed-ATR mice show a significant reduction in the floxed allele 8 weeks following hippocampal microinjection of AAV.Cre (**, p<0.001, n=7-9 per group). B,C Expression pattern of eGFP in the dorsal (B) and ventral (C) hippocampus 8 weeks following hippocampal microinjection of AAV.eGFP. Error bars indicate SEM.
Figure 2. Hippocampal ATR deletion inhibits hippocampal neurogenesis.

A) Cell Proliferation

B) Cell Survival

C) Immature Neurons

D) AAV.eGFP

E) AAV.Cre

F) AAV.eGFP

G) AAV.Cre
Figure 2. Hippocampal ATR deletion inhibits hippocampal neurogenesis. A,B AAV.Cre-injected mice exhibited significantly fewer BrdU-labeled cells within the subgranular zone as compared to AAV.eGFP-injected mice, both 24 hours (A, ***, p<0.0001, n=9-10 per group) and 4 weeks (B, ***, p<0.0001, n=12 per group) after BrdU injection. C Using sections from the same mice as in Figure 2a, DCX quantification revealed a significant decrease in immature neurons in the dentate gyrus of AAV.Cre-injected mice as compared with AAV.eGFP-injected mice (**, p<0.005, n=9-10 per group). D,E Representative photomicrographs of immunostaining for BrdU. Arrows point to BrdU-IR cells. F,G Representative photomicrographs of immunostaining for DCX. Error bars indicate SEM.
Figure 3. Hippocampal ATR deletion is associated with reduced anxiety in the elevated zero maze (EZM).

**A** AAV.Cre-injected mice spent significantly more time in the open arms of the maze, as compared to AAV.eGFP-injected mice (*, p < 0.01, n=5-7 per group).

**B** There was no difference between groups in the total distance traveled in the EZM. Error bars indicate SEM.
Figure 4. ATR deletion is associated with reduced anxiety. 

**Figure 4.** ATR deletion is associated with reduced anxiety. 

**A, B** ATR-deleted mice buried significantly fewer marbles during a 15-minute marble burying test. This effect was observed in both hippocampal specific ATR deletion (**, p<0.01, n=14-16 per group) and in the tamoxifen-inducible ubiquitous ATR deletion (**, p<0.001, n=6 per group). 

**C, D** ATR-deleted mice exhibited significantly shorter latencies to feed in the novel environment during the NIH test. This effect was observed both in hippocampal-specific ATR deletion (**, p<0.001, n=8 per group) and in the tamoxifen-inducible ubiquitous ATR deletion (**, p<0.001, n=11-13 per group). Error bars indicate SEM.
Figure 5. In the NIH paradigm, ATR-deleted mice exhibit altered antidepressant response.
**Figure 5.** In the NIH paradigm, ATR-deleted mice exhibit altered antidepressant response. **A** In a novel environment, AAV.Cre-injected mice exhibited a reduction in latency to consume peanut butter chips compared to AAV.eGFP-injected mice (#, p<0.0001, n=7-8 per group). There was a significant effect of chronic DMI treatment (12.5 mg/kg b.i.d., 24 days) only in the AAV.eGFP-injected mice (*, p<0.05). **B** CDP administered 30 minutes prior to novel testing reduces latency to feed in both control and ATR-deleted mice (##, p<0.0001 vs. saline-treated, n=8-10 per group). **C** DMI significantly reduces immobility in the FST in both control and ATR-deleted mice (##, p<0.0001 vs. saline-treated, n=8-10 per group). DMI was administered 24 hours (10 mg/kg), 5 hours (10 mg/kg), and 1 hour (20 mg/kg) prior to the 6-minute swim test. Immobility was measured over the final 4 minutes. **D** ATR deletion reduces cell proliferation in the dentate gyrus (###, p<0.001 vs. AAV.eGFP-treated, n=7-8 per group) Chronic DMI (12.5 mg/kg b.i.d., 24 days) has no effect on cell proliferation. Error bars indicate SEM.
Figure 6. Hippocampal CA1 dendritic spine density following DMI treatment.

A, B

Representative photomicrographs of Golgi impregnation staining to visualize dendritic arborization following saline (A) or DMI (B) treatment in AAV.eGFP-injected mice. C

Chronic DMI treatment significantly increased CA1 dendritic spine density in AAV.eGFP-injected mice (***, p < 0.001, n=30-42 per group). This effect was attenuated in AAV.Cre-injected mice. (*, p < 0.05). Error bars indicate SEM.
Figure S1. Hippocampal ATR deletion did not affect general locomotor activity or HPA axis activation following an acute stress.

Figure S1. Hippocampal ATR deletion did not affect general locomotor activity or HPA axis activation following an acute stress. **A, B** Floxed-ATR mice injected with AAV.Cre did not differ from AAV.eGFP-injected mice in a 60 minute locomotor activity test to measure ambulation (significant main effect of time, $F_{(11,198)}=26.84; p<0.0001$; no effect of AAV, $F_{(1,198)}=1.565; p=0.227$; no AAV x time interaction, $F_{(11,198)}=0.852; p=0.5884$, RMANOVA) and crossings (significant main effect of time, $F_{(11,198)}=25.23; p<0.0001$; no effect of AAV, $F_{(1,198)}=1.28; p=0.273$; no AAV x time interaction, $F_{(11,198)}=0.996; p=0.452$, RMANOVA). **C** ATR deletion did not affect serum corticosterone levels in response to an acute stressor (significant main effect of time, $F_{(3,39)}=140.7; p<0.0001$; no effect of AAV, $F_{(1,39)}=0.044; p=0.838$; no AAV*time interaction, $F_{(3,39)}=1.033; p=0.3884$, RMANOVA).
**Figure S2.** Fear conditioning is not altered by hippocampal ATR deletion.

**A** Acquisition of fear conditioning occurred following the first CS-US pairing, as measured by increased freezing across intervals (significant main effect of time, $F_{(4,88)}=38.3$, $p<0.0001$; no effect of AAV, $F_{(1,88)}=1.12$, $p=0.3013$; no AAV x time interaction, $F_{(4,88)}=0.159$, $p=0.958$, RMANOVA).

**B** When tested for contextual memory 24 hours following the training session, both AAV treatment groups exhibit increased freezing compared to baseline freezing levels prior to conditioning (significant main effect of time, $F_{(1,22)}=93.46$, ***$p<0.0001$; no effect of AAV, $F_{(1,22)}=0.115$, $p=0.738$; no AAV x time interaction, $F_{(1,22)}=0.213$, $p=0.649$, RMANOVA).

**C** Both AAV treatment groups respond similarly to a single CS exposure in a novel context (significant main effect of time, $F_{(1,22)}=20.18$, **$p<0.001$; no effect of AAV, $F_{(1,22)}=0.164$, $p=0.689$; no AAV x time interaction, $F_{(1,22)}=0.139$, $p=0.713$, RMANOVA).
Figure S3. ATR deletion does not induce impairments in spatial learning and memory in the Morris water maze.

Figure S3. ATR deletion does not induce impairments in spatial learning and memory in the Morris water maze. Paradoxically, ATR-deleted mice perform better than controls in locating the hidden platform. This subtle effect is an interesting area of exploration for future research, however it is beyond the scope and focus of the present paper. A Both AAV.eGFP and AAV.Cre-injected mice learned the location of the hidden platform, measured by reduced latency across 9 days (3 trials/day). RMANOVA revealed significant main effects of time \((F_{(8,152)}=44.04, p<0.0001)\), and AAV \((F_{(1,152)}=5.38, p<0.05)\) with no AAV x time interaction \((F_{(8,152)} = 0.494, p=0.859)\). B In the hidden platform probe test both treatment groups spent significantly greater time than chance in the target (North) quadrant (**p<0.0001, one sample T-test). C From a constant starting point, both treatment groups located the platform in a novel location in the reversal learning task. Additionally, ATR-deleted mice did not exhibit impairments in this task from a novel start location on the 7th day, suggesting intact spatial relational memory. RMANOVA revealed a significant main effect of time \((F_{(6,108)}=18.11, p<0.0001)\), a non-significant trend for an effect of AAV \((F_{(1,108)}=4.073, p=0.059)\), and no AAV x time interaction \((F_{(6,108)}=0.896, p=0.5)\).
Discussion

Since neurogenesis was first proposed to regulate response to antidepressant treatment (Jacobs et al., 2000), numerous publications have examined hippocampal neurogenesis in the context of depression. However, its precise role in the etiology and treatment of mood disorders remains controversial. In this study we showed that ATR is necessary for maintenance of the basal rate of hippocampal neurogenesis. Deletion of ATR from the hippocampus resulted in a partial inhibition of neurogenesis and reduced anxiety-like behavior in multiple mouse models of affective state. Antidepressant treatment was behaviorally ineffective in the NIH test following ATR deletion. In addition, the increased dendritic spine density induced by chronic treatment with DMI was severely attenuated following ATR deletion. These data suggest that capacity for increased neuronal plasticity in the hippocampus, rather than increased neurogenesis, may underlie the behavioral efficacy of DMI in the NIH test. A neurogenesis-independent effect of DMI in the NIH test is additionally supported by the observation that DMI reduced hyponeophagia in control mice without stimulating cell proliferation.

ATR is important for maintaining genomic integrity during DNA synthesis and proliferative failure is observed in its absence (Brown and Baltimore, 2000; Brown and Baltimore, 2003). No obvious behavioral changes are observed when ATR is deleted from differentiated cells in the forebrain (Ruzankina et al., 2007), suggesting that the behavioral alterations observed herein can be attributed to deletion of ATR from cells with proliferative potential following hippocampal AAV microinjection. The partial reduction in neurogenesis is likely related to the infection rate of the AAV in the neural stem and progenitor cells. Hypothetically, greater inhibition could be obtained by utilizing an approach that targets these cells with greater affinity. However, the
significant cellular and behavioral effects observed in this model of partially blocked neurogenesis reinforce the sensitivity of hippocampal functioning to changes in neurogenesis.

We observed a 45% reduction in hippocampal cell proliferation (Fig. 2a) and a similar reduction in the total number of 4-week old cells (Fig. 2b), 8 weeks following ATR deletion. Consistent with this finding, 54% fewer doublecortin-expressing cells were present in the hippocampus as a result of ATR deletion (Fig. 2c). Doublecortin is transiently expressed in immature neurons as they mature and migrate (Brown et al., 2003). These data indicate that ATR is necessary to maintain a normal rate of hippocampal neurogenesis. In a second model of ATR deletion that does not involve virus injection, we used a tamoxifen-inducible Cre transgene to delete ATR. In this model we observed a 30% reduction in cell proliferation as early as 5 weeks after ATR deletion. While this lesser reduction may be attributed to the abbreviated period between deletion and BrdU assay, the dose of tamoxifen and level of Cre induction may also be factors. Regardless, the reduced cell proliferation and reduced anxiety-like behavior observed in this second model used to delete ATR supports our finding that ATR plays a critical role in cell proliferation in the hippocampus.

ATR deletion resulted in reduced anxiety in multiple rodent behavioral tests, including the EZM (Fig. 3), marble burying test (Fig. 4a-b), and NIH test (Fig. 4c-d). These observations are at odds with studies associating reduced neurogenesis with increased anxiety in the elevated plus maze (Revest et al., 2009; Conboy et al., 2010) and light-dark box (Scobie et al., 2009), and finding no baseline change in hyponeophagia (Santarelli et al., 2003; Revest et al., 2009; Scobie et al., 2009). Interestingly, recent literature (Fuss et al., 2010a) suggests that intermediate levels of
neurogenesis are associated with low levels of anxiety in some paradigms, with exercise and irradiation, which increase and ablate neurogenesis, respectively, both associated with elevated anxiety. The anxiolytic phenotype observed in ATR-deleted mice, which exhibit levels of neurogenesis intermediate between basal levels and a complete ablation, appear to be in agreement with the aforementioned finding. Furthermore, though at the opposite end of the spectrum, our data is in line with studies demonstrating a correlation between increased neurogenesis and increased anxiety following voluntary exercise (Fuss et al., 2010b). This association between increased neurogenesis and increased anxiety is also observed in CREB-deficient mice (Gur et al., 2007).

The NIH test is considered an ethologically relevant test with strong predictive and construct validity (Dulawa and Hen, 2005). Our lab and others have previously demonstrated that latency to feed in the novel environment can be reduced by acute treatment with a benzodiazepine, as well as treatment with chronic, but not acute, antidepressant compounds (Dulawa and Hen, 2005, for review; Gur et al., 2007; Gundersen and Blendy, 2009). Our interest in examining the ATR mice in the NIH test was two-fold. First, baseline behavior in the novel environment provides a measure of anxiety. Second, this behavior is sensitive to chronic AD treatment that is thought to depend on increased neurogenesis. However, in studies where x-ray irradiation is used to ablate neurogenesis, there are discrepancies in the resulting effects on both baseline and antidepressant-mediated hyponeophagia (Santarelli et al., 2003; Holick et al., 2008; David et al., 2009). These conflicting findings may stem from variations in mouse strain, AD treatment, or hyponeophagia protocol, as some investigators utilize the novelty-suppressed feeding test, a variation of the NIH test. Additionally, reported effects of x-
ray irradiation on differentiation (Tada et al., 2000) and inflammatory response (Mizumatsu et al., 2003) have the potential to interfere with evaluation of hippocampal-modulated behaviors. Whether or not these factors confound the findings of studies using x-ray irradiation, confirmation by genetic methods in which neurogenesis is reduced or ablated will lend confidence to the interpretations and implications of altered neurogenesis and AD responsivity. To date, no one has reported whether a partial deficit in neurogenesis generated by genetic manipulation, such as that seen following ATR deletion, alters AD efficacy in a chronic exposure paradigm.

We observed a significant behavioral effect of DMI treatment in the NIH test only in our control mice (Fig. 5a). As baseline latencies were lower in the ATR-deleted mice, we ruled out a floor effect by testing the efficacy of a benzodiazepine, CDP, on latency in the novel test following ATR deletion. CDP significantly reduced latency to feed following ATR deletion (Fig. 5b). Both control and ATR-deleted mice exhibited reduced immobility time in the forced swim test after acute treatment (Fig. 5c), ruling out a more general deficit in response to DMI following ATR deletion.

Based on the behavioral observations in the NIH test, we hypothesized that DMI treatment in AAV.eGFP-injected mice would lead to increased neurogenesis, and that this effect of DMI would be attenuated in ATR-deleted mice. Interestingly, we observed no effect of DMI treatment on cell proliferation in either the control or ATR-deleted mice (Fig. 5d). While this was an unexpected observation, previous work has established that antidepressant effects on neurogenesis can be small in magnitude and depend on numerous factors including mouse strain and pretreatment (Balu et al., 2009; David et al., 2009). Furthermore, others have reported no effects of antidepressant treatment on
cell proliferation in rodents (Huang et al., 2008; Navailles et al., 2008; Petersen et al., 2009).

DMI reduces latency to feed without affecting cell proliferation, indicating that DMI’s effect on latency is through a neurogenesis-independent mechanism. To better understand this dissociation between the DMI’s effects on neurogenesis and behavior in the NIH paradigm, we sought to determine if DMI exerts other trophic effects in the hippocampus, and whether those effects may explain the lack of efficacy of DMI in the NIH paradigm following ATR deletion. To this end, we observed significantly increased CA1 dendritic spine density in control mice treated with DMI; this effect was greatly attenuated following ATR deletion (Fig. 6c). Dendritic spine density is enhanced by various external stimuli including learning (Moser et al., 1994), enrichment (Zhu et al., 2009), exercise (Stranahan et al., 2009) and antidepressant treatment; all stimuli which can have a positive influence on mood and emotional affect in depressed individuals. In agreement with work by others (Bessa et al., 2009), our results suggest that neuronal plasticity and/or remodeling, not increased neurogenesis, underlies the behavioral efficacy of antidepressant treatment.

As ATR is not necessary for the functioning of mature cells, attenuation of DMI-mediated increases in dendritic spine density in the CA1 region, an area of the hippocampus composed of mature neurons, hints at broad, circuit-wide changes in hippocampal function as a result of reduced neurogenesis. Immature neurons in the adult hippocampus exhibit unique properties including enhanced excitability and a lower threshold for the induction of long-term potentiation, indicative of enhanced potential for synaptic plasticity (Schmidt-Hieber et al., 2004). A reduction in the proportion of hippocampal neurons which exhibit these unique properties, as well a reduction in the
number of new neurons available to functionally integrate into the hippocampal circuitry, may interfere with homeostatic functioning of the hippocampus, indirectly rendering it less responsive to the changes normally induced by DMI. Consistent with this hypothesis, ablation of neurogenesis leads to a reduction in evoked perforant path responses and an increase in the amplitude of spontaneous network activity in the dentate gyrus in an in vivo model (Lacefield et al., 2010). It is not unreasonable to predict that changes in network activity in the dentate gyrus due to loss of young, plastic neurons might extend to interrupting circuitry function within other regions of the hippocampus. The importance of neurogenesis for the treatment of mood disorders may thus lie in the role of new neurons in maintaining homeostatic network activity within the hippocampus, and this concept is supported by evidence that fluoxetine depends on the plasticity of young neurons for its anxiolytic and antidepressant efficacy (Wang et al., 2008).

Hippocampal ATR deletion provides a valuable model for studying the behavioral changes associated with deficits in hippocampal neurogenesis. In future studies this model will be a useful tool for determining whether different classes of antidepressant compounds exhibit varying degrees of neurogenesis dependence. In addition, it is of interest to evaluate susceptibility to depressive-like behaviors in mouse models of depression such as chronic mild stress and chronic social defeat in the ATR-deleted mice. Further insight into the pathophysiology and treatment of depression should lead to the eventual development of more rapid-acting treatments with fewer undesired side effects.
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CHAPTER III

Low Neurogenesis Does Not Increase Susceptibility To Stress-Induced or Corticosterone-Induced Depressive Behaviors in Mice.
Abstract

Depression and mood disorders are associated with deficits in hippocampal size and function. Additionally, the birth of new neurons, or neurogenesis, in the hippocampus is thought to underlie the beneficial effects of certain antidepressant treatments in preclinical models. The relevance of hippocampal neurogenesis in the etiology of depression is less clear, with numerous studies suggesting reduced neurogenesis does not precipitate depressive-like phenotypes in rodents. This area of research is impeded by a poor understanding of the pathophysiology of depressive disorders in humans. In this study, we examined the effects of a partial blockade of adult hippocampal neurogenesis on basal and stress-induced affective behavior and neuroendocrine function. Neurogenesis was inhibited by approximately 50% in adult mice by hippocampal-specific deletion of ataxia-telangeictasia mutated and Rad-3 related (ATR) protein, a cell cycle checkpoint kinase essential for normal levels of neurogenesis. In male mice, reduced hippocampal neurogenesis was associated with an anhedonic phenotype in the sucrose preference test. However, in the learned helplessness paradigm, reduced neurogenesis did not influence the likelihood of developing learned helplessness or the extent of the escape deficit observed in susceptible mice. In addition, ATR deletion did not potentiate the effects of chronic corticosterone treatment. Consistent with current literature, these findings suggest reduced neurogenesis is likely not a prominent risk factor for mood disorders.
Introduction

Mood disorders, including major depressive disorder, dysthymic disorder and bipolar disorder, affect approximately 10% of adults in the U.S. each year (Kessler et al., 2003). Despite this high prevalence, the pathophysiology of mood disorders is poorly understood. Among individuals suffering from depressive disorders, a high level of heterogeneity is observed in the manifestation of symptoms as well as in the treatment efficacy and prognosis. This has led researchers and clinicians to suggest depressive disorders may be subdivided into a spectrum of subtypes with a diverse, multifactorial pathophysiology (Akiskal, 1989; Fagiolini and Kupfer, 2003).

The hippocampus has been extensively implicated in the etiology and treatment of mood disorders. Clinical studies in depressed individuals have demonstrated reduced hippocampal volume which is reversed by antidepressant treatment (Sheline et al., 1999). A twin study demonstrated that reduced hippocampal volume may be indicative of increased vulnerability to psychiatric illness (Gilbertson et al., 2002). In addition, the hippocampus plays a key regulatory role in the activity of the hypothalamic-pituitary-adrenal (HPA) axis. In response to stress, a major risk factor for depressive disorders (Kessler, 1997), HPA axis activation leads to the production of glucocorticoid stress hormones (Herman et al., 1996): cortisol in humans and corticosterone in rodents. Stress hormones activate glucocorticoid receptors within the hippocampus, leading to inhibitory feedback and suppression of subsequent glucocorticoid production (Herman et al., 1989; Jacobson and Sapolsky, 1991; van Haarst et al., 1997). Approximately 50% of depressed individuals exhibit HPA axis abnormalities (Gibbons, 1964).

A unique feature of the hippocampus is the continued formation of a substantial population of new neurons into adulthood, or neurogenesis (Altman and Das, 1965;
Ming and Song, 2005, for review). New neurons in the hippocampus have been extensively implicated in learning and memory processes as well as in mental health (Eisch et al., 2008, for review; Aimone et al., 2010, for review). Neurogenesis is subject to regulation by a variety of stimuli; factors such as stress (Gould et al., 1997; Tanapat et al., 1998) and drugs of abuse (Yamaguchi et al., 2004; Eisch et al., 2008) reduce hippocampal neurogenesis while learning (Patel et al., 1997; Gould et al., 1999), environmental enrichment (Kempermann et al., 1997; Kempermann et al., 1998), exercise (van Praag et al., 1999) and antidepressants (Malberg et al., 2000) can increase neurogenesis. Newborn neurons mature and functionally integrate into the hippocampal circuitry in approximately 4 weeks (van Praag et al., 2002), similar to the time necessary for antidepressants to exert their mood-improving actions in depressed individuals. A number of studies in which hippocampal neurogenesis is ablated by either x-ray irradiation or genetic manipulation suggest neurogenesis is necessary for the efficacy of some antidepressant drugs (Santarelli et al., 2003; Surget et al., 2008; David et al., 2009; Perera et al., 2011).

On the other hand, there is little direct evidence supporting reduced hippocampal neurogenesis in the pathophysiology of depression. Although reduced neurogenesis and depressive-like phenotypes arise in tandem following various chronic stressors including unpredictable chronic mild stress (Willner et al., 1987; Surget et al., 2008; Bessa et al., 2009), chronic corticosterone (Gourley et al., 2008; Murray et al., 2008), and social defeat stress (Berton et al., 2006; Czeh et al., 2007; Van Bokhoven et al., 2011), no one has demonstrated a requirement for reduced neurogenesis in the development of a depressive-like phenotype. However, we and others have shown inhibition of neurogenesis to be associated with altered anxiety-like behaviors (Bergami et al., 2008;
Revest et al., 2009; Onksen et al., 2011), which often present in a co-morbid, overlapping fashion with depressive symptoms.

We previously described the effects of Cre/lox-conditional deletion of ATR from the hippocampus of adult mice, specifically a partial reduction of hippocampal neurogenesis (Onksen et al., 2011). Here, we evaluated the effect of ATR deletion on rodent phenotypes considered to be representative of symptoms of depressive disorders, including anhedonia in a sucrose preference test, behavioral despair in a learned helplessness paradigm, and behavioral and endocrine effects of chronic corticosterone treatment.

**Materials & Methods**

**Animals** Mice homozygous for the Cre/lox-conditional allele of ATR (ATR^{f/f}) on a 129Sv/C57BL/6 background were generated as previously described (Ruzankina et al., 2007). ATR^{f/f} mice receiving hippocampal microinjection of AAV.Cre and having ATR deleted throughout the hippocampus are subsequently referred to as ATR^{ΔHipp}, while control mice are injected with AAV.eGFP and retain the ATR^{f/f} designation. Mice (1.5-5 months old, 20-35 gm) were housed in groups of 3-4 following stereotaxic surgery and during experimental paradigms, with exceptions noted. All behavioral experiments were conducted at least 5 weeks following stereotaxic surgery or tamoxifen treatment. Animals were maintained on a 12 hr light/dark cycle with food and water available ad libitum in accordance with the University of Pennsylvania Institutional Animal Care and Use Committee.
Experimental design

**Experiment 1.** Sucrose preference test \((n=17\text{ male } \text{ATR}^{ff}, 21\text{ male } \text{ATR}^{\Delta \text{Hipp}}, 13\text{ female } \text{ATR}^{ff}, 15\text{ female } \text{ATR}^{\Delta \text{Hipp}})\). To determine if mice with reduced hippocampal neurogenesis exhibit anhedonia, we performed a two-bottle choice sucrose preference test. Five weeks following adeno-associated virus (AAV) injection, mice were single-housed and given two weeks to acclimate to these new housing conditions. Mice were then acclimated to the presence of two drinking bottles for one week, and subsequently given a choice between water and a solution of 1% sucrose dissolved in water. Preference and total intake were analyzed for a 14 day period. This experiment was run in two separate cohorts of mice and data were pooled after determining that control groups did not differ from one another statistically.

**Experiment 2.** Learned helplessness. \((n=31\text{ male } \text{ATR}^{ff}, 29\text{ male } \text{ATR}^{\Delta \text{Hipp}})\). The learned helplessness paradigm was used to determine if mice with reduced hippocampal neurogenesis exhibit heightened susceptibility to the development of behavioral despair. Only males were used in this experiment, as we observed behavioral effects of ATR deletion in the sucrose drinking test only in male mice. Additionally, female mice are resistant to developing learned helplessness (Shors et al., 2007; Dalla et al., 2008). Mice were assigned to either a no shock control group or an inescapable shock (IS) group. Mice in the IS group were exposed to two 1-hour sessions of 120 inescapable footshocks while mice in the control group were placed in the training environment for the same length of time in the absence of footshocks. Subsequently, latency to escape from a series of 15 escapable footshocks was measured. The average escape latency during the final 5 escapable footshock trials was used for data analysis. A subset of mice \((n=20\)
ATR<sup>ff</sup>, 19 ATRA<sup>Hipp</sup>) were injected with bromodeoxyuridine (BrdU) 48 hours prior to the first training session to assess hippocampal cell genesis. These mice were killed 24 hours following the testing session and BrdU-positive cells were quantified by immunohistochemistry.

**Experiment 3.** Behavioral and endocrine response to chronic corticosterone. (n=20 ATR<sup>ff</sup>, 17 ATRA<sup>Hipp</sup>). Corticosterone pellets (21 day sustained release) were implanted to mimic chronic stress and to subsequently determine if reduced neurogenesis potentiates the effect of chronic stress on behavioral and endocrine endpoints. 26 days following pellet implantation, the forced swim test (FST) was performed. This consisted of a pre-swim session and a post-swim session. The antidepressant desipramine was given intraperitoneally three times in the 24 hour period between swim sessions. Both male and female mice were used and data was pooled after determining there was no sex effect in the FST. Three days following the forced swim test, in a subset of mice (n=8 male ATR<sup>ff</sup>, 8 male ATRA<sup>Hipp</sup>), we evaluated endocrine response to an acute stressor. Only males were used (to avoid sex hormone effects). A 15 minute restraint stress was performed and tail blood was collected prior to and at three time points following the restraint to measure serum corticosterone.

**Adeno-associated virus**  Adeno-associated viruses (AAV) expressing Cre recombinase (AAV2/9.CMV.PI.CRE, titer 2.84*10^{13} gc/ml) and eGFP (AAV2/9.CMV.eGFP, titer 3.74*10^{13} gc/ml) were generated by the University of Pennsylvania Vector Core. The expression cassette consists of the AAV2 terminal repeats flanking the CMV promoter-PI-Cre recombinase/eGFP sequences packaged into AAV9. The vectors were purified using
CsCl sedimentation method. Quantification of vector genome copies (gc) was performed by Q-PCR. AAVs were diluted in sterile PBS for microinjections.

**Hippocampal Injections**  
ATR<sup>ff</sup> mice (6-8 weeks) were anesthetized with isoflurane and secured in a stereotaxic frame (Kopf, Tujuna, CA). Holes were drilled bilaterally in the skull at sites of injection. Stereotaxic coordinates for the hippocampus are (from Bregma) anterior-posterior -2.1, lateral +/- 1.4, dorso-ventral -2.0, and anterior-posterior -2.9, lateral +/- 3.0, dorso-ventral -3.8. 0.5 ul of 1*10<sup>10</sup> gc/ul AAV was injected at each site through a 33 gauge needle on a 5 ul Hamilton syringe using a KDS310 Nano Pump (KD Scientific, Holliston, MA) mounted to the stereotaxic frame, at a rate of 0.1 ul/min. The needle remained in place for 4 additional minutes at each injection site. The skin was sutured and the animal recovered on a heating pad before returning to the home cage.

**Drugs**  
BrdU (Sigma) was dissolved in 0.9% saline immediately before use and injected intraperitoneally using a volume of 10 ml/kg. In the present study, 200 mg/kg BrdU was administered twice, 2 days prior to inescapable shock exposure in the learned helplessness paradigm, with injections separated by 8 hours. Corticosterone (5 mg/pellet, 21 day sustained release) or placebo pellets (Innovative Research of America, Sarasota, FL) were implanted subcutaneously. Each mouse in the corticosterone-treatment group was implanted with two 5 mg pellets to achieve the equivalent of 20 mg/kg/day. Desipramine (DMI, Sigma) was dissolved in 0.9% saline immediately before use and injected intraperitoneally using a volume of 10 ml/kg.
**Sucrose Preference**  Mice were single-housed 2 weeks prior to beginning the test. Singly-housed mice were acclimated to the presence of 2 water-containing drinking bottles in their home cage for 1 week. Subsequently, mice were presented with 1 bottle containing water and 1 bottle containing a 1% solution of sucrose (Fisher Scientific) dissolved in water. The position of each bottle within a cage was changed every 24 hours to mitigate bottle location preferences. Bottles were weighed every 24 hours to track consumption of both water and 1% sucrose. To accurately control for volume, two bottles were placed in a cage containing no mouse. Volume lost from these bottles averaged less than 5% of fluid intake and was subtracted from the daily intake prior to data analysis. Water and sucrose intake over every 2 days was averaged prior to analysis to account for any potential bottle position bias.

**Learned Helplessness**  Learned helplessness was performed as previously described (Berton et al., 2008). Training and testing was performed using an active avoidance system (Med Associates, Georgia, VT) consisting of a shuttle box divided into two compartments which are separated by an automated sliding door. The grid floor is capable of delivering scrambled shocks. Photocell detection tracks the location of the animal in the box. Training consisted of a one hour session in which mice in the inescapable shock (IS) group were confined to one compartment of the shuttle box and received 120 randomly distributed foot shocks (0.5 mA, 5 second duration). Training occurred once per day for two days. Animals in the no-shock control group were placed in the shuttle box for 1 hour in the absence of foot shocks. The testing session occurred 24 hours following the 2nd training session, and consisted of 15 escape trials spread over a 1 hour session. During each trial, a shock was delivered continuously and mice were
given the opportunity to escape by passing into the adjacent compartment through the
door, which would open at the beginning of each trial. The door closed following
successful escape. If mice failed to escape within 25 seconds, the shock was terminated
and the door was shut.

**Forced Swim Test**  Mice were placed in water (15 cm depth, 23°C) in plastic cylinders
(23 cm tall x 14 cm diameter) for 6 minutes. Using Viewpoint automated scoring
(Viewpoint, Champagne au Mont d'Or, France), duration of immobility was measured.
Mice were exposed to the swim tanks twice (pre-swim and swim test), with 24 hours
between exposures. Three injections of DMI were given during the inter-swim interval:
The first at 24 hours prior to the swim test (10 mg/kg), a second injection 5 hours prior
to the swim test (10 mg/kg), and a third injection 1 hour prior to the swim test (20
mg/kg).

**Restraint Stress**  Mice were placed in 50 mL Falcon conical vials for 15 minutes. Holes
were drilled at both ends to allow for breathing and to give access to the tail. Tail blood
(20 uL) was collected at 0, 15, 30, and 90 minutes. Blood was transferred directly into
tubes with chilled EDTA, centrifuged at 5,000 RPM for 10 minutes at 4°C, and serum was
used to measure corticosterone levels with a 125I Corticosterone RIA kit (MP Biomedicals,
Orangeburg, NY).

**Immunohistochemistry**  Mice were anesthetized with nembutol (10 mg/kg) and
transcardially perfused with cold 0.1 M phosphate-buffered saline (PBS) for 5 minutes,
followed by cold 4% paraformaldehyde (PFA) in PBS for 15 minutes. Brains were
postfixed overnight in PFA at 4°C and subsequently stored at 4°C in 30% sucrose. Brains were frozen on dry ice, sectioned coronally at a thickness of 40 µm, and transferred to PBS + 0.5% Sodium Azide at 4°C prior to processing for immunohistochemistry. For BrdU analysis, sections were wet-mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA) for stereological analysis using a modified version of the optical fractionator method (West et al., 1991). Mounted sections were incubated in 0.1 M citric acid, pH 6.0, for antigen retrieval. Subsequently, slides were incubated in trypsin, 2N HCl, then primary antibody (mouse anti-BrdU, 1:200, Becton Dickinson) with 0.5% Tween 20 overnight. On the second day, slides were incubated for 1 hour in secondary antibody (biotinylated anti-mouse IgG, Vector Laboratories), 1 hour in avidin-biotin-HRP (1:100, Vector Laboratories), and labeled cells were visualized using diaminobenzidine (Sigma). PBS-washed slides were then counterstained and coverslipped with Permount. BrdU-IR cells in every 9th section of the hippocampus were counted with a 100x oil immersion lens, omitting cells in the uppermost focal plane. Cells within clusters were counted by distinguishing nuclear borders while focusing down through the tissue using an objective with a narrow depth of focus. For proliferation counts, a cell is included in the subgranular zone of the dentate gyrus if it is touching, or within two cells of the granule cell layer.

**Statistical Analysis** Sucrose drinking data was analyzed by two-factor ANOVA with time as a repeated factor. Escape latency and cell proliferation data were analyzed by two-factor ANOVA. Two-factor ANOVAs were performed using GraphPad Prism 5 and Bonferroni’s post-tests were applied where appropriate. Stress-induced serum
corticosterone and forced swim test immobility were analyzed by three-factor ANOVA using JMP software. All data are reported as mean ± SEM.

**Results**

**Experiment 1: Sucrose preference**

Male ATR$^{\Delta \text{Hipp}}$ mice exhibit a marked reduction in preference for a 1% sucrose solution (Fig. 1a) compared to ATR$^{\text{ff}}$ controls (gene main effect, $F_{(1,216)}=12.01$, $p<0.01$; time main effect, $F_{(6,216)}=5.92$, $p<0.0001$). This difference is not observed following ATR deletion in female mice (Fig. 1b; gene main effect, $F_{(1,156)}=0.255$, $p=0.618$; time main effect, $F_{(6,156)}=5.5$, $p<0.0001$). ATR deletion does not affect total fluid consumption in males (Fig. 1c; gene main effect, $F_{(1,216)}=2.12$, $p=0.154$; time main effect, $F_{(6,216)}=3.35$, $p<0.01$) or females (Fig. 1d; gene main effect, $F_{(1,156)}=1.82$, $p=0.189$; time main effect, $F_{(6,156)}=9.86$, $p<0.0001$).

**Experiment 2: Learned helplessness**

*Learned helplessness.* Based on sucrose preference findings, we examined whether the anhedonic phenotype observed in males would translate to susceptibility in the learned helplessness paradigm, a rodent model of behavioral despair. Following 2 days of exposure to repeated, inescapable footshocks, latency to escape from escapable footshocks was measured. Mice were exposed to 15 escapable footshock trials during a 1 hour test session. The average escape latency of the last 5 trials was calculated (Fig. 2a). A significant main effect of inescapable shock was observed ($F_{(1,56)}=27.77$, $p<0.0001$). There was no effect of ATR deletion on average escape latency.
Variability was observed in the effect of inescapable shock treatment; some mice were susceptible to developing learned helplessness while others performed similar to no-shock controls. ATR deletion did not influence the proportion of mice that were susceptible.

Cell genesis. A subset of mice in the learned helplessness experiment received two injections of BrdU (200mg/kg, IP) two days prior to inescapable footshock exposure to evaluate cell genesis in the hippocampus (Fig. 2d). As expected, there was an overall effect of ATR deletion to reduce BrdU-labeled cells \((F_{(1,32)}=35.24, p<0.001)\). Exposure to inescapable footshocks did not have a significant effect on this outcome measure \((F_{(1,32)}=0.296, p=0.59)\). In addition, no correlation was observed between escape latency and cell genesis (data not shown).

Experiment 3: Behavioral and endocrine response to chronic corticosterone

Forced swim test. Because stress is a major risk factor in the pathophysiology of depression, we sought to determine if reduced neurogenesis exacerbates the effects of chronic stress. To do this, we examined the effects of ATR deletion on behavioral and endocrine endpoints following chronic corticosterone treatment. 21 day extended-release pellets were implanted to deliver 20 mg/kg/day corticosterone to mimic the effects of chronic, mild stress. To assess the behavioral effect of chronic corticosterone, immobility in the FST was measured 26 days following pellet implantation (Fig. 3a). Increased immobility in the FST is interpreted as behavioral despair. Three-factor ANOVA revealed a near-significant effect of corticosterone pellet to increase immobility \((F_{(1,27)}=3.69, p=0.066)\) and, as a positive control, a significant effect of the antidepressant desipramine (DMI) to reduce immobility \((F_{(1,27)}=9.09, p<0.01)\). There
was not a significant gene effect ($F_{(1,27)}=0.035$, $p=0.85$) and no significant interactions were observed, indicating that ATR deletion does not alter the expected effects of prior corticosterone experience and of antidepressant treatment on immobility in the FST.

Restraint stress. We previously showed that the partial reduction of neurogenesis observed following hippocampal ATR deletion is not associated with altered corticosterone response to an acute restraint stress in naïve mice. Here, we evaluated the effect of chronic corticosterone on subsequent response to an acute restraint stress. 28 days following corticosterone pellet implantation, mice were subjected to a 15 minute restraint stress and tail blood was collected at 0, 15, 30 and 90 minutes to measure serum corticosterone (Fig. 3b). Analysis by three-factor ANOVA revealed a significant main effect of time (time main effect, $F_{(3,63)}=90.37$, $p<0.0001$; gene main effect, $F_{(1,63)}=0.04$, $p=0.839$; pellet main effect, $F_{(1,63)}=0.16$, $p=0.693$) and a significant time x pellet interaction ($F_{(3,63)}=9.48$, $p<0.0001$). Bonferroni post-tests to compare corticosterone treatment at the 4 time points indicated that prior corticosterone pellet experience blunted the corticosterone response to an acute stressor at the 15 minute and 30 minute time points. ATR deletion did not influence the effect of chronic corticosterone exposure on acute stress response.
Figure 1. Sucrose preference and fluid intake.

Figure 1. Sucrose preference and total fluid intake. (A,B) Sucrose consumption as a percent of total fluid intake across 14 days. Males exhibit reduced preference following ATR deletion (A, *p<0.01). (C,D) ATR deletion does not affect total fluid intake during the sucrose preference test. Error bars represent SEM.
**Figure 2.** Learned helplessness. (A) Mice in the inescapable shock (IS) group exhibit significantly greater escape latencies during the escape trials compared to the control no-shock (NS) mice (***p<0.0001 vs. NS group). (B) Mice exposed to inescapable shock do not differ from no-shock controls in the number of BrdU-positive cells in the hippocampus. There is a gene main effect such that ATR deletion results in significantly reduced BrdU-immunoreactive (IR) cells (#p<0.001, two-way ANOVA). Error bars represent SEM.
Figure 3. Behavioral and endocrine consequences of chronic corticosterone.

A. Forced Swim Test

B. Restraint Stress
**Figure 3.** Behavioral and endocrine consequences of chronic corticosterone. (A) Immobility during the final 4 minutes of a 6 minute swim test is graphed. Desipramine (DMI) reduced immobility and prior corticosterone (Cort) pellet experience increased immobility. There was no effect of ATR deletion within any of the four pellet/antidepressant conditions. N = 4-5 per treatment group. (B) Serum cort is plotted at 4 time points. Restraint stress occurred between the 0 and 15 minute time points. Prior corticosterone pellet experience resulted in significantly reduced cort at the 15 and 30 minute time points compared to placebo pellet (*, p<0.05). There was no significant effect of ATR deletion. N = 4 per treatment group. Error bars represent SEM.
Discussion

In this study we explored the possibility that a partial reduction in hippocampal neurogenesis may result in depressive-like phenotypes in mice. We previously showed an approximately 50% reduction in hippocampal neurogenesis following deletion of ATR from this brain region and additionally observed alterations in anxiety-like behaviors and in response to antidepressant treatment. Thus, we sought to determine if these effects extended to phenotypes representative of the symptoms of depression.

ATR deletion resulted in a significantly reduced preference for a 1% sucrose solution in male mice (Fig. 1). This effect was not observed in female mice, and was not due to a change in total fluid intake. Reduced sucrose preference in rodents is an indicator of anhedonia, a key symptom of depression. Preference for this palatable solution can be reduced by chronic stress and subsequently brought back to normal levels by treatment with antidepressants (Katz, 1982; Willner et al., 1987; Papp et al., 1991; Muscat et al., 1992). Various forms of chronic stress can lead to reduced neurogenesis as well as reduced sucrose preference in both mice (Aonurm-Helm et al., 2008; Goshen et al., 2008) and rats (Veena et al., 2009). A survey of the literature to identify studies in which neurogenesis was knocked down through x-ray irradiation, cytotoxicity, or genetic manipulation revealed just two studies in rats demonstrating no effect of reduced neurogenesis on sucrose preference (Bessa et al., 2009; Noonan et al., 2010), suggesting chronic stress reduces neurogenesis and sucrose preference independently of each other. We are not aware of studies in mice evaluating sucrose preference following a non-stress induced reduction of neurogenesis, leaving open the possibility that reduced neurogenesis blunts hedonic response. Alternatively, it can not be ruled out that the anhedonic phenotype we observe is not due to an unintended
effect of single housing (Hellemans et al., 2004; Wei et al., 2007). Recent work from Belforte and colleagues found single housing to potentiate anxiogenic and anhedonic phenotypes in male mutant mice with a conditional knockout of NMDA receptor subunit NR1 from cortical and hippocampal GABAergic interneurons (Belforte et al., 2010). Mice in our study were single-housed two weeks prior to the start of the sucrose drinking task. This mild stressor may have acted synergistically to induce an anhedonic phenotype in ATR-deleted mice. Greater sensitivity to single-housing among male mice in this particular measure of anhedonia would explain why we do not see an effect in females. A possible stress effect of single housing suggests reduced neurogenesis alone may not be sufficient to induce anhedonia in group-housed animals.

We next evaluated behavioral despair in the learned helplessness paradigm following ATR deletion. This paradigm, first described by Overmier and Seligman (1967), models depression through exposure to unpredictable and unavoidable shocks which lead to deficits in subsequent coping behavior. Due to multiple reports that females are resistant to the development of behavioral deficits in the learned helplessness paradigm (Shors et al., 2007; Dalla et al., 2008) only males were used for this portion of the study. We observed a significant effect of IS on escape latency during the avoidance test (Fig. 2a), such that IS resulted in a phenotype indicative of behavioral despair, or a lack of effort to escape from an avoidable footshock. In addition, among IS-treated mice, some were susceptible to developing learned helplessness while others were resistant and behaved similar to no shock control mice in the avoidance test. This phenomenon is responsible for the relatively large standard error observed among IS treatment groups compared to the NS control groups. This individual susceptibility has been observed previously in both rats and mice (Vollmayr et al., 2003; Chourbaji et al.,
2005). Consistent with other studies investigating the relationship between hippocampal neurogenesis and learned helplessness (Vollmayr et al., 2003; Reif et al., 2004; but see Ho and Wang, 2010), we did not observe an effect of ATR deletion on the proportion of mice susceptible to developing learned helplessness. In addition, escape latency and cell genesis did not correlate, further supporting the conclusion that rate of neurogenesis does not directly influence the development of learned helplessness. As expected, ATR deletion resulted in an overall effect of reduced cell genesis (Fig. 2b).

In the third experiment we examined the effects of reduced hippocampal neurogenesis on chronic exposure to low dose corticosterone. Exogenous corticosterone induces behaviors reminiscent of depression and anxiety, similar to those observed following chronic mild stress (Murray et al., 2008; Zhao et al., 2008; David et al., 2009; Crupi et al., 2010). The dose used in the present study (20 mg/kg/day, 21 day release) has been shown to induce increased immobility in the forced swim test (FST) and tail suspension test without inhibiting cell proliferation (Murray et al., 2008; Zhao et al., 2008). This dose was selected to determine if corticosterone levels that have a sub-threshold effect on cell proliferation produce more severe depressive-like symptoms on a background of low neurogenesis. We found chronic corticosterone exposure to increase immobility in a subsequent FST (Fig. 3a), indicative of increased behavioral despair. However, ATR deletion did not alter the corticosterone pellet’s effects on immobility in the FST. In addition to this behavioral outcome measure, we evaluated endocrine response to an acute stressor by measuring serum corticosterone during an acute restraint stress following chronic corticosterone pellet exposure. Prior corticosterone exposure blunted the subsequent response to an acute stress experience (Fig. 3b). Similar to the FST, ATR deletion did not increase the severity of the corticosterone
pellet’s effects. From these findings we can conclude that the detrimental effects of chronic exposure to heightened circulating corticosterone are not exacerbated by reduced basal levels of neurogenesis.

In conclusion, while susceptibility to depression is strongly influenced by numerous factors such as social stress, environmental stress and genetics, neurogenesis does not appear to exert a significant influence over the development of depressive-like behavior in rodents. Mechanistically, new neurons are essential for detecting and encoding subtle contextual changes, a process also referred to as pattern separation (Clelland et al., 2009; Sahay et al., 2011). Based on this, Perera and colleagues have postulated that suppressed neurogenesis, occurring during periods of stress, may result in uncoupling of affect and external context during negative mood states. Inability to recognize subtle reductions in stress due to poor pattern separation may then lead to persistence of a negative mood that is no longer reflective of the external context, precipitating a depressive disorder (Perera et al., 2008). Due to the diverse pathophysiology and varied treatment responsiveness across the population of depressed individuals, it is indeed possible that neurogenesis plays an role in certain cases of clinical depression. However, current methods of examining depressive-like behavior in pre-clinical models have not yet revealed reduced neurogenesis to be a significant risk factor.
References


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CHAPTER IV

Mice Deficient in Hippocampal Neurogenesis Do Not Develop an Anxiety Phenotype Following Wheel Running

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Abstract

Exercise, specifically voluntary wheel running, is a potent stimulator of hippocampal neurogenesis in adult mice. In addition, exercise induces behavioral changes in numerous measures of anxiety in rodents. However, the physiological underpinnings of these changes are poorly understood. To investigate the role of neurogenesis in exercise-mediated anxiety, we examined the cellular and behavioral effects of voluntary wheel running in mice with a reduction in hippocampal neurogenesis, achieved through conditional deletion of ataxia telangiectasia-mutated and rad-3 related protein (ATR), a cell cycle checkpoint kinase necessary for normal levels of neurogenesis. Following hippocampal microinjection of an adeno-associated virus expressing Cre recombinase to delete ATR, mice were exposed to four weeks of voluntary wheel running and subsequently evaluated for anxiety-like behavior. Wheel running resulted in increased cell proliferation and neurogenesis, as measured by bromodeoxyuridine and doublecortin, respectively. Wheel running also resulted in heightened anxiety in the novelty-induced hypophagia, open field, and light-dark box tests. However, both the neurogenic and anxiogenic effects of wheel running were attenuated following hippocampal ATR deletion, suggesting increased neurogenesis is an important mediator of exercise-induced anxiety.
Introduction

Exercise is associated with a wide array of health benefits (van Praag, 2008, for review). In addition to the physical benefits, exercise can improve cognition (van Praag et al., 1999; Van der Borght et al., 2007; Winter et al., 2007; Nichol et al., 2009), alleviate symptoms of neurodegenerative diseases (Tillerson et al., 2003; Nichol et al., 2009), as well as aid in recovery from mood disorders (Blumenthal et al., 1999; Babyak et al., 2000; Duman et al., 2008).

Rodents exhibit a strong penchant for voluntary wheel running, which activates brain reward pathways (Brene et al., 2007) and is used as a model to investigate the mechanisms underlying the benefits of exercise in humans. Voluntary running results in a robust increase in hippocampal neurogenesis in adult mice. Indeed, running has been shown to increase proliferation of neural progenitor cells (Kronenberg et al., 2003), and increase both the number and percentage of newborn cells which become mature neurons (van Praag et al., 1999). Running and neurogenesis have been directly correlated, both within and across mouse strains, further highlighting the potent influence of exercise on hippocampal neurogenesis (Allen et al., 2001; Rhodes et al., 2003; Clark et al., 2011). A multitude of changes are implicated in the therapeutic effects of exercise, including heightened synaptic plasticity (van Praag et al., 1999; Farmer et al., 2004), angiogenesis (Van der Borght et al., 2009) and growth factor expression (Neeper et al., 1996; Kitamura et al., 2003). As evidenced by the importance of newborn hippocampal neurons in cognition and mood regulation (Sahay et al., 2011; Surget et al., 2011), neurogenesis is an additional mechanism which may contribute to the therapeutic effects of exercise.
In light of clinical evidence for the mood-improving effects of exercise (Deslandes et al., 2009, for review), we set out to investigate the underlying mechanisms associated with these beneficial effects. A number of studies have investigated the effects of voluntary wheel running on mood and anxiety-related behaviors in rodents, the results of which are inconsistent. While some studies find decreased anxiety (Dishman et al., 1996; Salam et al., 2009), others demonstrate increased anxiety (Burghardt et al., 2004; Fuss et al., 2010). Yet others demonstrate either increased or decreased anxiety depending on the specific anxiety measure used and the timing of the measurement in relation to time after removal from running wheels (Binder et al., 2004; Duman et al., 2008). Reports of heightened anxiety following wheel running appear counterintuitive to the beneficial effects of exercise on mood in humans. However, a recent study demonstrating that running-induced anxiety was prevented by x-ray irradiation, a technique to ablate neurogenesis (Fuss et al., 2010), supports a role for neurogenesis in the development of exercise induced-anxiety.

We previously showed partial suppression of hippocampal neurogenesis following hippocampal deletion of ATR, a cell cycle checkpoint kinase (Onksen et al., 2011). Here, we explored the possibility that deletion of ATR from the hippocampus would result in resistance to the neurogenic effects of voluntary exercise and examined the behavioral implications of that resistance.

**Materials & Methods**

**Animals** Mice homozygous for the Cre/lox-conditional allele of ATR (ATR^{f/f}) on a 129SV/C57BL/6 background were generated as previously described (Ruzankina et al.,
ATR^{f/f} mice receiving hippocampal microinjection of AAV.Cre and having ATR deleted throughout the hippocampus are subsequently referred to as ATR^{ΔHipp}, while control mice are injected with AAV.eGFP and retain the ATR^{f/f} designation.

Mice (1.5-4 months old, 20-35 gm) were housed in groups of 3-4 following stereotaxic surgery and subsequently single-housed at least one week prior to being placed into cages containing running wheels. Cages with running wheels (Mini Mitter, Bend OR) measured 20x36 cm with an 11.5 cm diameter wheel mounted to the cage top. Wheel rotations were monitored continuously via VitalView (Mini Mitter). Sedentary control animals were housed in identical cages with immobilized wheels.

All behavioral experiments were conducted on adult males and females at least 6 weeks following stereotaxic surgery. Animals were maintained on a 12 hr light/dark cycle with food and water available ad libitum in accordance with the University of Pennsylvania Institutional Animal Care and Use Committee.

**Experimental Design** Two cohorts of ATR^{f/f} mice were used in this study. Mice in both cohorts received hippocampal micro-injections at 8 weeks of age. For a schematic of the experimental design see Figure 1a. Mice in cohort #1 were used for the novelty-induced hypophagia (NIH) test and were therefore single housed and trained to consume peanut butter chips in their home cages prior to being placed in cages containing running wheels. Cohort #2 was used to evaluate behavior in the open field, marble-burying and light-dark box tests (separated by 3 days). All behavioral testing was performed during the 4^{th} week of wheel running. All mice were injected with 200 mg/kg bromodeoxyuridine (BrdU, Sigma) 24 hours following the last behavioral test and subsequently perfused 24 hours following BrdU injection. Mice had ad libitum access to
running wheels until they were perfused (except when removed from the cages for behavioral testing). Data from both cohorts was pooled to examine effects of AAV injection and voluntary running on neurogenesis.

**Adeno-associated virus** Adeno-associated viruses (AAV) expressing Cre recombinase (AAV2/9.CMV.PI.CRE, titer 2.84*10^{13} gc/ml) and eGFP (AAV2/9.CMV.eGFP, titer 3.74*10^{13} gc/ml) were generated by the University of Pennsylvania Vector Core. The expression cassette consists of the AAV2 terminal repeats flanking the CMV promoter-PI-Cre recombinase/eGFP sequences packaged into AAV9. The vectors were purified using CsCl sedimentation method. Quantification of vector genome copies (gc) was performed by Q-PCR. AAVs were diluted in sterile PBS for microinjections.

**Hippocampal Injections** ATR^{ff} mice (6-8 weeks) were anesthetized with isoflurane and secured in a stereotaxic frame (Kopf, Tujuna, CA). Holes were drilled bilaterally in the skull at sites of injection. Stereotaxic coordinates for the hippocampus are (from Bregma) anterior-posterior -2.1, lateral +/- 1.4, dorso-ventral -2.0, and anterior-posterior -2.9, lateral +/- 3.0, dorso-ventral -3.8. 0.5 ul of 1*10^{10} gc/ul AAV was injected at each site through a 33 gauge needle on a 5 ul Hamilton syringe using a KDS310 Nano Pump (KD Scientific, Holliston, MA) mounted to the stereotaxic frame, at a rate of 0.1 ul/min. The needle remained in place for 4 additional minutes at each injection site. The skin was sutured and the animal recovered on a heating pad before returning to the home cage.
**Immunohistochemistry** Mice were anesthetized with nembutol (10 mg/kg) and transcardially perfused with cold 0.1 M phosphate-buffered saline (PBS) for 5 minutes, followed by cold 4% paraformaldehyde (PFA) in PBS for 10 minutes. Brains were postfixed overnight in PFA at 4°C and subsequently stored at 4°C in 30% sucrose. Brains were frozen on dry ice, sectioned coronally at a thickness of 40 µm, and transferred to PBS + 0.5% Sodium Azide at 4°C prior to processing for immunohistochemistry.

For BrdU analysis, sections were wet-mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA) for stereological analysis using a modified version of the optical fractionator method (West et al., 1991). Mounted sections were incubated in 0.1 M citric acid, pH 6.0, for antigen retrieval. Subsequently, slides were incubated in trypsin, 2N HCl, then primary antibody (mouse anti-BrdU, 1:200, Becton Dickinson) with 0.5% Tween 20 overnight. On the second day, slides were incubated for 1 hour in secondary antibody (biotinylated anti-mouse IgG, Vector Laboratories), 1 hour in avidin-biotin-HRP (1:100, Vector Laboratories), and labeled cells were visualized using diaminobenzidine (Sigma). PBS-washed slides were then counterstained and coverslipped with Permount. BrdU-IR cells in every 9th section of the hippocampus were counted with a 100x oil immersion lens, omitting cells in the upper-most focal plane. Cells within clusters were counted by distinguishing nuclear borders while focusing down through the tissue using an objective with a narrow depth of focus. For proliferation counts, a cell is included in the subgranular zone (SGZ) of the dentate gyrus if it is touching, or within two cells of the granule cell layer (GCL).

For doublecortin (DCX) analysis, free-floating sections of a 1/9 series through the hippocampus were blocked for 1 hour with 3% Normal Horse Serum, 0.5% Tween 20 and 0.2% Triton in PBS (blocking solution). Sections were then incubated for 72 hours
at 4°C in primary antibody (Goat anti-DCX, Santa Cruz #8066) diluted 1:500 in blocking solution. Sections were washed and incubated for 1 hour in secondary antibody (Horse anti-goat, Vector Laboratories) diluted 1:200 in blocking solution. Sections were then washed and treated with 0.75% H$_2$O$_2$ for 20 minutes prior to incubation in avidin-biotin-HRP (1:200, Vector Laboratories). Labeled cells were visualized using nickel-enhanced diaminobenzidine. Free-floating sections were mounted onto slides and dried overnight before dehydrating and coverslipping with Permount. DCX-IR cells in every 9th section were counted with a 100x oil immersion lens, omitting cells in the upper-most focal plane. All cells located within the GCL and SGZ were counted.

**Novelty-induced Hypophagia** For 1 week prior to the training period, and through the experiment, mice were single housed. On 11 consecutive training days, mice were exposed to highly palatable food (peanut butter chips, Nestle, Glendale CA) in a plastic dish in their home cage. On each training day mice were acclimated to the presence of a plastic cage divider for 1 hour, consistent with previous NIH studies (Gur et al., 2007; Onksen et al., 2011). Peanut butter chips were then placed in the cage for 15 minutes and latency to initiate feeding was measured. Subsequent to training, mice were placed in cages containing running wheels and left for 28 days. On days 25 and 26, a home cage test was performed in which mice were removed from wheel-containing cages and placed into a standard mouse cage, allowed to acclimate to the cage and divider for 1 hour, and latency to feed was subsequently measured. On day 27, latency to feed in a novel environment was measured. Mice were removed from wheel-containing cages, placed for 1 hour in the same standard mouse cage with divider that was used for home cage tests, and subsequently placed in a novel, anxiety provoking environment where
latency to feed was measured. The novel environment consisted of an empty cage with no bedding, set inside a white box with a bright light and a novel odor (pine sol). Following each test session, mice were returned to their running wheel-containing cages.

**Open Field** Open field behavior was monitored in a white box measuring 41x35 cm in dim light conditions. Activity was recorded by an overhead camera and analyzed with TopScanLite 2.0 (Clever Sys, Inc., Reston, VA) Parameters assessed were time spent and distance traveled in the center and outer zones. The center zone was defined as 25% of the total area.

**Light-Dark Box** The light-dark box consisted of two adjacent chambers, connected by a 5x5 cm opening. Each chamber measured 17x20 cm. The dark chamber was black and covered. The light chamber was white and illuminated with bright light. Each test session lasted 5 minutes and was initiated by placing the mouse in the corner of the light chamber. Behavior was recorded by an overhead camera and analyzed with TopScanLite 2.0. Parameters assessed were transitions between the two chambers, time spent in each chamber, and distance traveled in the light chamber.

**Statistical analysis** Statistical analyses were performed with GraphPad Prism 5 and JMP software. Data are reported as mean ± SEM. Studies involving two variables were analyzed by two-way ANOVA, with time as a repeated measure where appropriate. Bonferroni post hoc tests were performed to compare individual treatment groups. Data
from the NIH test was analyzed by three-factor ANOVA to account for gene, exercise, and test environment.

Results

**ATR deletion attenuates the neurogenic effect of voluntary wheel running**

To determine if ATR deletion confers resistance to the neurogenic effects of wheel running, we microinjected AAV.Cre or AAV.eGFP into the hippocampus of ATR\(^{ff}\) mice and assigned them to either the running or the sedentary condition. Mice were single housed in running wheel-containing cages 6 weeks following AAV injection. Running distance plateaued in the 2\(^{nd}\) week in both ATR\(^{ff}\) and ATR\(^{\Delta\text{Hipp}}\) mice (Fig. 1b), which is consistent with other studies involving wheel running (Clark et al., 2009). No differences were observed in wheel running distance between treatment groups (ATR\(^{ff}\), 7.73±2.49 km/day; ATR\(^{\Delta\text{Hipp}}\), 8.31±2.14 km/day; gene main effect, \(F_{(1,72)}=0.404, p=0.53\); time main effect, \(F_{(1,72)}=16.57, p<0.001\)).

Following 28 days of running, immunohistochemical analysis was performed to measure cell proliferation and number of immature neurons. BrdU (200 mg/kg) was injected 24 hours prior to perfusing the animals. Two-way ANOVA of BrdU-positive cells in the hippocampus (Fig. 2a) revealed main effects of gene (\(F_{(1,50)}=28.89, p<0.0001\)) and exercise (\(F_{(1,50)}=23.33, p<0.0001\)), as well as a significant interaction (\(F_{(1,50)}=5.26, p<0.05\)). *Post tests* indicate that wheel running resulted in a significant increase in cell proliferation in ATR\(^{ff}\) runners compared to all other treatment groups (+89% vs. ATR\(^{ff}\) sedentary, +206% vs. ATR\(^{\Delta\text{Hipp}}\) sedentary, +102% vs. ATR\(^{\Delta\text{Hipp}}\) runners, each \(p<0.001\)). Unlike ATR\(^{ff}\) runners, ATR\(^{\Delta\text{Hipp}}\) runners did not exhibit significantly increased cell proliferation.
proliferation compared to their sedentary counterparts. In addition, as previously observed, ATR deletion resulted in significantly reduced cell proliferation among sedentary mice (-38%, p<0.05). Two-way ANOVA of doublecortin (DCX)-positive cells in the hippocampus (Fig. 2b) revealed significant main effects of gene ($F_{(1,51)}=32.65$, p<0.0001) and exercise ($F_{(1,51)}=39.73$, p<0.0001), as well as a significant interaction ($F_{(1,51)}=10.08$, p<0.01). Similar to our BrdU observations, post tests indicated DCX expression was significantly increased in ATR$^{ff}$ runners compared to all other groups (+172% vs. ATR$^{ff}$ sedentary, +406% vs. ATR$^{A\text{Hipp}}$ sedentary, +146% vs. ATR$^{A\text{Hipp}}$ runners, each p<0.001). ATR$^{A\text{Hipp}}$ runners did not exhibit significantly increased DCX-expressing cells compared to their sedentary counterparts. Post tests did not show a significant effect of ATR deletion among sedentary mice. However, we previously demonstrated an effect of ATR deletion to reduce DCX-expressing cells, so we therefore performed a Student’s $t$-test comparing sedentary ATR$^{ff}$ and ATR$^{A\text{Hipp}}$ mice to verify this effect had again been achieved, and indeed it had (-46%, $t_{(30)}=4.00$, p<0.001). Taken together, BrdU and DCX data indicate that mice lacking hippocampal ATR exhibit a reduced neurogenic response to wheel running.

**Running-induced anxiety in the NIH test is attenuated in ATR$^{A\text{Hipp}}$ mice**

We recently observed reduced feeding latency in a novel environment in mice with reduced levels of neurogenesis (Onksen et al., 2011). The NIH paradigm consists of training mice to consume a palatable food in their home cage, and subsequently measuring their latency to consume this same food in a novel, anxiety provoking environment. Thus, reduced neurogenesis may contribute to reduced anxiety in this particular paradigm. As others have demonstrated heightened anxiety as a result of
voluntary exercise, we sought to determine whether our voluntary wheel running paradigm would result in increased anxiety in the NIH test, and whether this anxiogenic phenotype would be altered by reduced neurogenesis.

Six weeks following hippocampal microinjection of AAV to delete ATR, mice were single housed and subsequently trained to consume peanut butter chips in their home cage. No differences were observed in training performance between ATR\textsuperscript{f/f} and ATR\textsuperscript{ΔHipp} mice (Fig. 3a), as measured by two-way ANOVA of feeding latency on days 6-11, with day as a repeated measure (day main effect, $F_{(5,150)}=15.91$, $p<0.0001$; gene main effect, $F_{(1,150)}=0.339$, $p=0.565$). Training was followed by 28 days of housing in running-wheel-containing cages.

On the 25\textsuperscript{th} and 26\textsuperscript{th} days of running, mice were given access to peanut butter chips in a home cage environment. Latency was measured on the 2\textsuperscript{nd} home environment exposure. Our unpublished observations indicated exercise to be anxiogenic when mice are initially removed from running wheels and tested for feeding latency in a home cage. Thus, we measured feeding latency during the second day of home cage feeding, during which all treatment groups performed similarly (Fig. 3b). Normalization of home cage behavior allows for clear interpretation of any anxiety phenotypes observed in the novel test. On the 27\textsuperscript{th} day of running, feeding latency in a novel, anxiety-provoking environment was measured.

Three-factor ANOVA of feeding latency revealed significant main effects of gene ($F_{(1,51)}=7.64$, $p<0.01$), exercise ($F_{(1,51)}=7.94$, $p<0.01$) and environment ($F_{(1,51)}=23.31$, $p<0.001$), in addition to a significant gene x exercise x environment interaction ($F_{(1,51)}=24.29$, $p<0.001$). Post test comparisons indicated significantly greater latency in the ATR\textsuperscript{f/f} runners compared to all other treatment groups in the novel environment (Fig.
3b, p<0.05). Among ATR\(^{Δ\text{Hipp}}\) mice, there was no difference in latency between runners and their sedentary counterparts in the novel environment (Fig. 3b), indicating ATR deletion blocked the anxiogenic effects of running on feeding latency.

**Running-induced anxiety in an open field is attenuated in ATR\(^{Δ\text{Hipp}}\) mice**

Voluntary wheel running results in increased anxiety in an open field test, as measured by reduced time spent in the center zone. This anxiogenic phenotype was attenuated in ATR\(^{Δ\text{Hipp}}\) mice. Two-way ANOVA of time spent in the center zone (Fig. 4a) revealed a near-significant effect of exercise (\(F_{(1,24)}=3.67, p=0.067\)) and a near-significant exercise x gene interaction (\(F_{(1,25)}=2.5, p=0.12\)). Bonferroni post tests to compare individual treatment groups indicated significantly reduced time spent in the center zone in ATR\(^{ff}\) runners compared to their sedentary counterparts (-67%, \(t_{(13)}=2.445, p<0.05\)). ATR\(^{Δ\text{Hipp}}\) runners did not differ from either sedentary control group. While the reduced distance traveled in the center zone in ATR\(^{ff}\) runners appears to be blunted in ATR\(^{Δ\text{Hipp}}\) mice, two-way ANOVA of distance traveled in the center zone (Fig. 4b) revealed significant main effects of exercise (\(F_{(1,24)}=5.646, p<0.05\)) and gene (\(F_{(1,24)}=5.232, p<0.05\)), but no exercise x gene interaction (\(F_{(1,24)}=0.58, p=0.45\)). Similar results were obtained when measuring total distance traveled in the open field (Fig. 4c; exercise main effect \(F_{(1,24)}=5.75, p<0.05\); gene main effect \(F_{(1,24)}=9.77, p<0.01\), exercise x gene interaction \(F_{(1,24)}=0.06, p=0.81\)), suggesting exercise exerts an overall anxiogenic effect on exploratory behavior while ATR deletion exerts an overall anxiolytic effect on exploratory behavior in the open field.
Running-induced anxiety in the light-dark box test is attenuated in ATR^ΔHipp mice

As an additional measure of anxiety, we evaluated behavior in the light-dark box following voluntary wheel running. Two-way ANOVA revealed a significant main effect of exercise ($F_{(1,25)}=12.82$, $p<0.01$) on light-to-dark transitions (Fig. 5a). There was no effect of gene ($F_{(1,25)}=0.004$, $p<0.95$) and no exercise x gene interaction ($F_{(1,25)}=0.19$, $p<0.67$) on this outcome measure, suggesting the effect of exercise on transitions is not dependent on a robust stimulation of neurogenesis. Analysis of distance traveled in the light compartment revealed a trend towards an anxiogenic effect of running which was blunted in ATR^ΔHipp mice (Fig. 5b). Two-way ANOVA revealed a near-significant main effect of exercise ($F_{(1,25)}=3.97$, $p=0.057$) and a near-significant exercise x gene interaction ($F_{(1,25)}=3.105$, $p=0.09$). Post tests revealed a significant decrease in distance traveled in the light compartment in ATR^{f/f} runners compared to their sedentary counterparts ($p<0.05$). This effect was not apparent in ATR^ΔHipp mice. No effects of running or ATR deletion were observed on time spent in the light compartment (exercise main effect $F_{(1,25)}=0.01$, $p<0.92$; gene main effect $F_{(1,25)}=1.14$, $p=0.30$; exercise x gene interaction $F_{(1,25)}=0.001$, $p=0.97$). Reduced distance traveled in the light compartment in the absence of any changes in total time spent in the compartment may be indicative of reduced exploration.
Figure 1. Experimental design

A Two cohorts of mice were used in this study, the first for novelty-induced hypophagia and the second for marble burying, open field and light-dark box tests. Both cohorts were injected with AAV, single-housed approximately 5 weeks later, and then placed in running wheels for 4 weeks. Behavioral testing occurred in the final week of running. BrdU (200 mg/kg, IP) was injected 24 hours following the last behavioral test and mice were perfused 24 hours following BrdU injections. B No significant differences were observed in running distance between ATR^{ff} and ATR^{ΔHipp} mice. Error bars represent SEM.
**Figure 2.** ATR$^{\Delta\text{Hipp}}$ mice exhibit deficits in exercise-induced neurogenesis.

**A** Voluntary exercise resulted in a significant increase in BrdU-expressing cells in ATR$^{fl/fl}$ mice (***, $p<0.001$ vs. ATR$^{fl/fl}$-S, ATR$^{\Delta\text{Hipp}}$-S and ATR$^{\Delta\text{Hipp}}$-R groups, n=11-16 per treatment group). Among sedentary controls, ATR deletion results in significantly reduced BrdU-expressing cells (#, $p<0.05$ vs. ATR$^{fl/fl}$-S group). 

**B** Voluntary exercise resulted in a significant increase in DCX-expressing cells in ATR$^{fl/fl}$ mice (***, $p<0.001$ vs. ATR$^{fl/fl}$-S, ATR$^{\Delta\text{Hipp}}$-S and ATR$^{\Delta\text{Hipp}}$-R groups, n=11-16 per treatment group). In addition, among sedentary controls, ATR deletion results in reduced DCX-expressing cells ($\gamma$, $p<0.001$ vs. ATR$^{fl/fl}$-S group by Student’s t-test). Error bars represent SEM. Abbreviations: S - sedentary; R - runner.
Figure 3. Running-induced anxiety in the NIH test is attenuated in ATR^{ΔHipp} mice

**Figure 3.** Running-induced anxiety in the NIH test is attenuated in ATR^{ΔHipp} mice. **A** No differences were observed between AAV treatment groups in home cage NIH training prior to placement in running wheels. **B** In the home test all mice exhibited comparable feeding latencies. On the novel test day ATR^{ff} runners exhibited significantly greater latency compared to each of the other treatment groups (***, p<0.001 vs. ATR^{ff}-S, ATR^{ΔHipp}-S and ATR^{ΔHipp}-R, n=4-8 per group). Error bars represent SEM. Abbreviations: S - sedentary; R - runner.
Figure 4. Running-induced anxiety in an open field is attenuated in ATR\textsuperscript{ΔHipp} mice.

A  
Time in Center

B  
Distance Traveled in Center

C  
Total Distance Traveled
**Figure 4.** Running-induced anxiety in an open field is attenuated in ATR$^{ΔHipp}$ mice. **A** ATR$^{iff}$ runners spent less time in the center zone compared to their sedentary counterparts (*, p<0.05 vs. ATR$^{iff}$-S). **B,C** Running exerts a main effect of reduced distance traveled in the center and reduced total distance traveled compared to sedentary mice. ATR deletion exerts a main effect of increased distance traveled in the center zone and increased total distance traveled compared to control ATR$^{iff}$ mice. There are no significant differences between individual groups. N=6-8 per group. Error bars represent SEM. Abbreviations: S - sedentary; R - runner.
Figure 5. Running-induced anxiety in the light-dark box is attenuated in ATR\(^{\Delta \text{Hipp}}\) mice.

A. Transitions

B. Distance in Light Compartment

C. Time in Light Compartment
**Figure 5.** Running-induced anxiety in the light-dark box is attenuated in ATR^{ΔHipp} mice.

A Running exerts a main effect of reduced transitions compared to sedentary mice. B ATR^{ff} runners explored the light compartment less than their sedentary counterparts (**, p<0.01 vs. ATR^{ff}-S), as measured by distance traveled in the light compartment. This effect is not observed in ATR^{ΔHipp} mice. C No differences were observed in total time spent in the light compartment. N=6-8 per group. Error bars represent SEM. Abbreviations: S - sedentary; R - runner.
Discussion

Exercise is associated with many health benefits in humans, ranging from the cognitive to the physiological (van Praag, 2008). Preclinical research has established exercise as a potent enhancer of hippocampal neurogenesis (van Praag et al., 1999), which may underlie its health benefits. Interestingly, exercise leads to changes in anxiety-like behavior in rodents. The potential role of neurogenesis in modulating anxiety-related behaviors following exercise is only now being explored. In this study, we utilized a transgenic mouse in which Cre recombinase-inducible deletion of ATR from the hippocampus of adult mice leads to reduced levels of hippocampal neurogenesis. Our previous characterization of hippocampal ATR deletion examined effects on basal neurogenesis only. Here, we found that ATR deletion attenuated the neurogenic effect of wheel running. Additionally, wheel running resulted in heightened anxiety in the novelty-induced hypophagia, open field, and light-dark box tests. Many of the anxiety phenotypes induced by wheel running were absent following ATR deletion. While our findings are in agreement with work by others (Fuss et al., 2010), there are conflicting reports in the literature regarding effects of exercise on anxiety (Dishman et al., 1996; Binder et al., 2004; Burghardt et al., 2004; Duman et al., 2008; Salam et al., 2009). These discrepancies may arise from a multitude of factors including variation in housing conditions and exercise parameters. However, the most relevant factor is likely differences in running distance and neurogenic response to running across mouse strains (Clark et al., 2011), which will subsequently influence behavioral outcome measures.

We utilized a 4 week, ad libitum access wheel running paradigm, as we previously observed increased cell proliferation and BDNF mRNA in the hippocampus in
naïve ATR\textsuperscript{ff} mice using this paradigm (J.L.O., unpublished observations). ATR deletion did not alter running behavior, as measured by running distance across 4 weeks (Fig. 1b). Wheel running exerted a potent stimulatory effect on neurogenesis in ATR\textsuperscript{ff} mice, as indicated by increased BrdU and DCX immunostaining to measure cell proliferation and immature neurons, respectively (Fig. 2). This effect of wheel running was attenuated in ATRΔHipp mice; levels of neurogenesis in ATRΔHipp runners were comparable to those of sedentary ATR\textsuperscript{ff} controls. Our neurogenesis data suggests ATRΔHipp mice retain some neurogenic capacity in response to a strong stimuli. However, the overall magnitude of the increase remains well below that which is observed in the control group, allowing for investigation of the causal role of excessive neurogenesis in the behavioral changes observed following exercise.

To identify behavioral implications of a blunted neurogenic response to exercise, we examined the anxiety state of the animals. We first examined behavior in the NIH paradigm following exercise. Both ATR\textsuperscript{ff} and ATRΔHipp mice exhibited similar feeding latencies during the training period, prior to exercise (Fig 3a). In the novel environment, we observed a large increase in latency to feed in ATR\textsuperscript{ff} runners compared to sedentary ATR\textsuperscript{ff} controls (Fig. 3b). This increase was not apparent in the ATRΔHipp runners, suggesting heightened neurogenesis may underlie the effect observed in ATR\textsuperscript{ff} mice. Because ATR deletion does not influence feeding latency in the home cage test, it is possible that the role of neurogenesis in exercise-induced anxiety is context-dependent and is most relevant in novel environments. Previously, we observed a statistically significant reduction in latency to feed in a novel environment following ATR deletion (Onksen et al., 2011), whereas here we observe only a trend in the NIH test (Fig. 3b) and ATR deletion alone does not reduce all measures of anxiety examined in the present
study. We hypothesize that the single-housing conditions necessary for the wheel running studies result in alterations to anxiety state (Kwak et al., 2009) such that certain differences are less likely to be observed.

In addition to the NIH paradigm, we examined anxiety-like behavior in the open field and light-dark box tests. In the open field test (Fig. 4), ATR\textsuperscript{ff} runners spent less time in the center zone compared to their sedentary counterparts, indicative of increased anxiety. This effect was not observed in ATR\textsuperscript{ΔHipp} runners. When distance traveled in the open field was analyzed, we found exercise to reduce distance traveled, indicative of increased anxiety or reduced exploratory behavior. Additionally, ATR deletion increased distance traveled, indicative of reduced anxiety or increased exploratory behavior, consistent with our previous work showing reduced anxiety following ATR deletion (Onksen et al., 2011). In the light-dark box test (Fig. 5), running resulted in fewer transitions between the compartments. This effect was present in both ATR\textsuperscript{ff} and ATR\textsuperscript{ΔHipp} runners. Wheel running resulted in reduced distance traveled in the light compartment in ATR\textsuperscript{ff} mice. This effect was completely blocked in ATR\textsuperscript{ΔHipp} mice, and was not an effect of total time spent in the light, which was similar across groups. Among previous reports using the light-dark or dark-light tests, some report changes in both transitions and time spent in the light compartment as measures of anxiety (Chaouloff et al., 1997; Frye et al., 2008; Varadarajulu et al., 2011), while others report one or the other (Binder et al., 2004; Correa et al., 2008; Fuss et al., 2010; Pilhatsch et al., 2010). Variability may be dependent on differences in mouse strain or parameters of the testing environment, including size and lighting conditions. Thus, we analyzed transitions, distance traveled in the light compartment, and time in the light compartment in our mice.
Taken together, this data from multiple anxiety tests suggests mice with increased neurogenesis exhibit heightened anxiety. This phenotype may also be interpreted as reduced exploration or slower habituation to a unfamiliar environment. This alternative explanation is supported by observations of reduced distance traveled in the open field (Fig. 4) and in the light compartment of the light-dark box (Fig. 5) following wheel running in ATR\textsuperscript{f/f} mice. Because increased neurogenesis is associated with heightened cognition in rodent models (Sahay et al., 2011), the phenotype exhibited by ATR\textsuperscript{f/f} runners may be due to increased awareness of novel surroundings and subsequent caution in exploring the environment.

In conclusion, we have shown here that hippocampal neurogenesis is an important determinant of some anxiety-like behaviors in mice. Findings of heightened anxiety would seem at odds with clinical evidence for the beneficial effects of moderate exercise. However, the data presented herein, in addition to recent literature highlighting the prevention of running-induced anxiety by x-ray irradiation (Fuss et al., 2010), strengthens the hypothesis that neurogenesis directly influences the anxiety-like behavior induced by running. As further explanation of exercise effects which seem counterintuitive to the beneficial effects observed in humans, running distances achieved by rodents may not be representative of the exercise behavior that is beneficial to the treatment of mood and anxiety disorders in humans and may indicate that too much neurogenesis can be detrimental to certain behaviors (Saxe et al., 2007). Indeed, moderate exercise is recommended as therapy for depressed mood in humans (Greer and Trivedi, 2009), often in conjunction with pharmacotherapy, and excessive exercise can have negative consequences (Peluso and Guerra de Andrade, 2005; Purvis et al., 2010; Czepluch et al., 2011). Thus, while rodent models of exercise are a valuable tool.
for studying neurogenesis, caution must be exercised in applying the results of unlimited voluntary wheel running to the human population. Future work should focus on developing rodent models of exercise which accurately mirror moderate exercise in humans, and on understanding the mechanisms through which newborn neurons in the hippocampus influence anxiety-like behaviors. This is especially pertinent in the context of efforts to develop therapeutic compounds that stimulate hippocampal neurogenesis for the treatment of mood disorders.
References


CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

The overall aim of this dissertation was to examine the role of hippocampal neurogenesis in the etiology and treatment of mood and anxiety disorders. Post-mortem and fMRI studies of depressed patients’ brains suggest the hippocampus is relevant to these mental illnesses (Webster et al., 2002; Gaughran et al., 2006; Lorenzetti et al., 2009) and the process of neurogenesis within the dentate gyrus of the hippocampus is of particular interest. However, current methods utilized to study hippocampal neurogenesis in rodents are invasive and cannot be employed in depressed patients. This limitation has led the field to rely heavily on rodent models. Our examination of the influence of neurogenesis in mood disorders was accomplished through a transgenic mouse model of suppressed hippocampal neurogenesis. ATR deletion from the adult mouse was previously shown to induce aging-like phenotypes due to a depletion of stem and progenitor cells (Ruzankina et al., 2007); however a specific effect of ATR deletion on neurogenesis in the dentate gyrus of the hippocampus was not previously evaluated. We demonstrated a partial suppression of hippocampal neurogenesis following ATR deletion from the hippocampus of the adult mouse. Spatial and temporal regulation of ATR deletion was accomplished through hippocampal microinjection of an AAV expressing Cre-recombinase.

Other approaches to inhibiting hippocampal neurogenesis, including x-ray irradiation, produce a near-complete ablation of new neurons, and are thus valuable for evaluating the function of new neurons in certain cases. While the dynamics of hippocampal neurogenesis are poorly understood in humans, the partial suppression
observed following ATR deletion may be more representative of a pathological state than a complete ablation would be. Using ATR deletion to suppress hippocampal neurogenesis, we found changes in behaviors representative of mood and anxiety disorders and alterations in response to antidepressant treatment. These insights are a valuable contribution to the field of hippocampal neurogenesis and psychiatric illness. The following paragraphs contain a more extensive discussion of the findings and implications of this dissertation, and highlight areas of interest for future investigation.

In characterizing the cellular effects of hippocampal ATR deletion, we observed an approximately 50% reduction in cell proliferation and in the number of immature neurons present 8 weeks following AAV injection. This partial suppression is in contrast to the near-complete ablation that can be achieved with other methods. The partial effect observed here may be due to the affinity of the AAV to neural stem/progenitor cells. A future study using either an alternative viral approach or a transgenic approach, such as Cre recombinase expression through the Nestin promoter, would shed light on whether ATR deletion is a viable approach for obtaining an even greater suppression of neurogenesis. Subsequently, one could compare the behavioral effects of partial and complete suppression within the same transgenic line.

ATR deletion resulted in reduced anxiety in the novelty-induced hypophagia, marble burying, and zero maze tests. These observations are at odds with some studies reporting increased anxiety in mice with reduced neurogenesis (Revest et al., 2009; Scobie et al., 2009; Conboy et al., 2010). However, our observation of reduced anxiety makes sense in the context of models in which heightened anxiety is associated with heightened neurogenesis (Gur et al., 2007; Fuss et al., 2010), including our own work highlighting the anxiogenic effect of exercise and blockade of this effect in ATR-deleted
mice. In addition, hippocampal lesion studies have reported reduced anxiety in social, exploratory, and novelty tasks (Bannerman et al., 2002). As anxiety can be parsed into these various aforementioned categories, a more extensive analysis of which types of anxiety measures are affected by ATR deletion may improve understanding of how neurogenesis modulates anxiety.

We observed a lack of antidepressant response in the NIH paradigm following ATR deletion, similar to work by others suggesting antidepressants work though stimulating neurogenesis (Santarelli et al., 2003). However, in our hands we did not observe an effect of DMI on cell proliferation in control animals and did find the effects of DMI on CA1 dendritic spine density to be attenuated in ATR-deleted mice, indicative of a blunted capacity for synaptic plasticity. Work by others has indicated that neuronal remodeling is the relevant underlying factor in antidepressant efficacy, rather than neurogenesis (Bessa et al., 2009). It is possible that these two phenomena, neurogenesis and neuronal plasticity/remodeling, are intimately connected. Future studies to examine broad hippocampal circuitry function in ATR-deleted mice, using methods such as voltage-sensitive dye imaging and electrophysiology, may shed light on whether a partial suppression of neurogenesis can alter homeostatic functioning of the hippocampus, as was demonstrated following x-ray irradiation to ablate neurogenesis (Lacefield et al., 2010). In addition, we examined only one antidepressant, DMI, a tricyclic which mainly inhibits norepinephrine reuptake and, to a lesser extent, serotonin reuptake. Antidepressants with more potent effects on serotonergic signaling, as well as antidepressants with non-monoaminergic mechanisms, may exhibit a differential requirement for hippocampal neurogenesis in modulating hyponeophagia behavior.
While many reports argue that reduced hippocampal neurogenesis is not sufficient to induce depressive-like behaviors, we were compelled to consider the potential for depressive-like phenotypes, as ATR deletion is a novel approach to inhibiting neurogenesis and results in a unique, partial suppression. We observed anhedonia in male ATR-deleted mice, an effect that was absent in females. Additional research should exclude possible metabolic differences after ATR deletion, as well as rule out the possibility that single-housing played a role in the appearance of the anhedonic phenotype. Future studies to delve further into the sex-specific effect might include ovariectomizing females to determine the contribution of sex hormones to the females’ resiliency to anhedonia in the sucrose drinking task. Subsequent to observing an anhedonic phenotype in males, we explored the possibility that they would be more susceptible to helplessness in the learned helplessness paradigm. The proportion of ATR-deleted mice which developed helplessness did not differ from that of control mice. In addition, the average escape latency in avoidance trials following inescapable shock was not affected by ATR deletion. Finally, in the learned helplessness studies, we did not observe any correlation between escape latency and cell genesis, indicating neurogenesis does not influence the extent of learned helplessness or the likelihood that a particular mouse will be susceptible.

As stress is a major risk factor for depression, we examined whether ATR deletion would potentiate the effects of chronic corticosterone treatment. The consequences of this treatment, thought to mirror some of the effects of chronic stress, were not exacerbated by ATR deletion. Low dose corticosterone administered through a 21-day sustained-release pellet (20 mg/kg/day), to mimic chronic mild stress, resulted in increased immobility in the forced swim test and reduced corticosterone response to an
acute stressor. No interaction between the corticosterone treatment and ATR deletion was observed. The restraint stress study was performed in males only. As sex differences were observed in the sucrose drinking task, it is possible that males and females are differentially susceptible to certain depressive-like phenotypes. Implanting corticosterone pellets in females and subsequently measuring response to an acute stressor might thus yield a potentiating effect of ATR deletion in females, even though it is not observed in the males.

In addition to examining baseline behaviors following ATR deletion, we examined a stimuli which normally exerts a potent neurogenic effect in rodents, voluntary wheel running. Our interest in wheel running stems from two sources. First, wheel running exerts a potent influence on neurogenesis and alters anxiety state. Second, in humans exercise is recommended as a treatment for mood disorders, often in conjunction with pharmacotherapy. These two points present an interesting contrast; a stimulus which improves mood clinically can increase anxiety in pre-clinical models, an effect contradictory to the treatment of psychiatric disease. Further, in the NIH test, chronic wheel running in place of chronic antidepressant treatment does not result in the antidepressant-like effect of reduced hyponeophagia. One significant difference between voluntary wheel running in rodents and voluntary exercise in humans is the extent of the exercise. Mice can run more than 10 km/day when given unlimited wheel access; accounting for the difference in size between a mouse and human, this is significantly farther than a human runs in a given day when partaking in moderate, voluntary exercise. Thus, the rodent model of ad libitum access to voluntary wheel running may not be the ideal model for understanding precisely the mechanisms through which
exercise improves mood in humans. Nonetheless, wheel running is a valuable tool for understanding how neurogenesis influences anxiety.

The potent neurogenic effect of wheel running is attenuated in ATR-deleted mice, such that ATR-deleted runners exhibit levels of cell proliferation and neurogenesis similar to a sedentary control animal. Additionally, wheel running resulted in anxiety in the NIH paradigm, marble burying test, open field, and light-dark box, in agreement with work by others (Burghardt et al., 2004; Fuss et al., 2010b; Fuss et al., 2010). In some of our outcome measures, ATR deletion blocked the anxiogenic effect of running. In addition to increased anxiety, the behavior observed following exercise is reminiscent of reduced exploration and slowed habituation to novel situations. As increased neurogenesis is often associated with heightened cognition, the anxiety phenotype observed in control runners and blocked in ATR-deleted runners may be due to increased alertness and awareness in a novel environment. Additional studies should be designed to parse these potentially independent phenomena, pure anxiety versus heightened alertness and caution in response to novel surroundings in mice with elevated hippocampal neurogenesis.

In conclusion, our studies to examine mood and anxiety disorders in the ATR transgenic mouse support a complex and essential role for hippocampal neurogenesis in these psychiatric diseases. The high prevalence of these disorders and lack of optimal treatment options support the continued exploration and characterization of novel drug development targets, such as hippocampal neurogenesis, in hope of identifying compounds which improve symptoms with greater efficacy and fewer unwanted side effects.
References


