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Nicola D. Hanson

Effects of stress and psychoactive drugs on adult hippocampal neurogenesis in the rat

Nicola D. Hanson
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Program in Neuroscience

Charles B. Nemeroff, MD, PhD
Advisor

Michael J. Owens, PhD
Committee Member

Michael Davis, PhD
Committee Member

Jay M. Weiss, PhD
Committee Member

Ronald S. Duman, PhD
Committee Member

Accepted:

Lisa A. Tedesco, PhD — Date
Dean of the Graduate School

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Nicola D. Hanson
BS, Emory University 2000

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Abstract

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By

Nicola D. Hanson

The subgranular zone of the dentate gyrus is one of only two sites of adult neurogenesis in mammalian species. It is widely hypothesized to be downregulated by psychological stress and upregulated by antidepressant drugs and to play a role in the psychopathology of major depression. However, increasing evidence of inconsistencies and complications surrounding this hypothesis has led to the questioning of its accuracy and support for a more nuanced understanding of the relation of hippocampal neurogenesis to other stress-regulated systems of the brain. The experiments presented here show that the processes involved in hippocampal neurogenesis, primarily proliferation of neural progenitor cells (NPC), are not susceptible to alteration by either psychological stress or antidepressant drugs under all circumstances. Robust and prolonged stress experiences, including restraint, tail shock, and social defeat, failed to affect the proliferation rate of NPCs and short or long term survival of resulting newborn cells. The selective serotonin reuptake inhibitors (SSRIs) fluoxetine and escitalopram, the CRF_1 antagonist R121919, and the atypical antipsychotics paliperidone and ziprasidone also failed to affect neurogenesis in any measure examined. Chronic treatment with the mood stabilizer lithium caused a significant increase in NPC proliferation but no alteration in survival rate of newborn cells. Additionally, NPC proliferation in adolescent animals showed no greater responsivity to either stress or drug treatment than was seen in young adults. These results, seemingly in conflict with the accepted model of the association of adult hippocampal neurogenesis with stress and antidepressant drug response, are part of an emerging, more complex grasp of the role neuroplasticity plays in stress response.

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1: Background

The first evidence of newly generated neurons in adulthood was reported in 1965, when Altman and Das used tritiated thymidine to label proliferating cells in the subgranular zone (SGZ) of the rat dentate gyrus and their daughter cells which became morphologically identical to mature granule cells (Altman and Das 1965). Later studies confirmed that these new cells were indeed neurons (Kaplan and Hinds 1977). The presence of the highly polysialated form of neural cell adhesion molecule (PSA-NCAM or NCAM-H), which is only found in immature neurons, provided additional evidence that neurogenesis occurs in the adult dentate gyrus (Seki and Arai 1993). The existence of adult-generated neurons in the human hippocampus was demonstrated some years later (Eriksson, Perfilieva et al. 1998).

The subventricular zone (SVZ) of the lateral ventricle is now known to be a second site of adult neurogenesis, producing neuroblasts which travel along the rostral migratory stream (RMS) to differentiate into interneurons of the olfactory bulb (reviewed in (Garcia-Verdugo, Doetsch et al. 1998)). As these cells are not responsive to stress or psychoactive drugs, they will not be addressed further here. Additionally, some reports show a small number of adult-generated GABAergic interneurons in the neocortex of rodents and non-human primate, although negative reports regarding this region are equally as compelling (reviewed in (Cameron and Dayer 2008). Strong evidence argues that this is unlikely to occur in humans (Bhardwaj, Curtis et al. 2006).

Timeline and markers of hippocampal neurogenesis

The subgranular zone (SGZ) of the dentate gyrus contains Type-1 radial-glia-like stem cells, which express the astrocytic marker glial fibrillary acidic protein (GFAP) as well as nestin. Their population size and proliferation rate are relatively constant; these are likely the true stem cells of the region. These cells exhibit morphology of radial glia and divide asymmetrically, resulting in Type-2 daughter cells. Type-2 and, later, Type-3 cells are GFAP-negative and much more proliferative than Type-1 cells, and their proliferation rates are acutely variable. Type-2 cells express the intermediate filament protein nestin and, as they develop, the microtubule-associated protein doublecortin (DCX) and the polysialated form of neural cell adhesion molecule (PSA-NCAM). Type-3 cells continue to express doublecortin but no longer express nestin. Type-3 cells are generally referred to as neuroblasts, while Type-2 are called lineage-determined progenitor cells or neural progenitor cells (NPCs). During the Type-3 stage, cells shift the orientation of new processes from horizontal (parallel to the granule cell layer) to vertical (perpendicular) and begin radial migration into the granule cell layer. When the maturing cell exits the cell cycle, it reaches the Type-4 cell stage, about three days after the original division. These immature granule cells express calcium-binding protein calretinin, and also begin to express neuronal nuclei protein (NeuN) and send out axons toward the CA3 region. Survival of the newborn cell population is largely regulated during this stage via variable rates of apoptosis. 2-3 weeks after exiting the cell cycle, they cease expressing calretinin and instead express calbindin, a marker of mature granule cells. A total of 4-7 weeks are required for newborn cells to become functionally

indistinguishable from the older granule cell population (Kempermann, Jessberger et al. 2004; Zhao, Deng et al. 2008).

Dividing cell populations have traditionally been studied using nucleotide analogues: originally [^{3}H]-thymidine, and later bromodeoxyuridine (BrdU) and, less often, iododeoxyuridine (IdU) or chlorodeoxyuridine (CldU). When made available via systemic injection, these molecules are incorporated into replicating DNA in place of thymidine, resulting in labeled cells which can be visualized using autoradiography or immunocytochemistry. Some concerns have been raised that BrdU might have cytotoxic effects or otherwise disrupt normal proliferation or maturation processes, but careful studies have not shown any deleterious actions of BrdU doses well above those commonly used in labeling protocols (Hancock, Priester et al. 2009). One notable disadvantage of BrdU use is that it only labels cells which are in S-phase at the time of the pulse (BrdU is metabolized and/or excreted within approximately two hours following injection), while progenitor cells in the dentate gyrus have been determined to have a cell cycle time of 24.7 hours (Cameron and McKay 2001). Labeled cells therefore indicate a proportion of the entire population of cycling cells, and so changes in number of BrdU-labeled cells accurately represent changes in the total number of currently cycling cells, but a single pulse will not give a full count of the true cycling population. Many investigators attempt to solve this problem by using repeated pulses, maintaining BrdU availability over at least a full cell cycle, but because BrdU-labeled DNA is passed on to daughter cells, this adds the complication of allowing those cells labeled early in the availability period to complete mitosis following labeling, resulting in doubling of the

labeled cell number, whereas those labeled late in the period will not have time to complete mitosis and will only result in single labeled cells. This distorts the makeup of the targeted cell population. The number of cells labeled by a single pulse of a nucleotide analogue peaks at one week, marking the shift of most of the population from the proliferative Type-2 and -3 stages to the nonproliferative Type-4, wherein cells are subjected to a high rate of apoptosis. At four weeks post-labeling, virtually all labeled cells in the granule cell layer express neuron-specific enolase (NSE); the small number which express GFAP are likely Type-1 progenitor cells (Cameron, Woolley et al. 1993).

In recent years, the endogenous protein marker Ki-67 has emerged as an alternative labeling method of proliferating cells. This protein is expressed in the nucleus during all phases of the cell cycle, although its function is as yet undetermined (Kee, Sivalingam et al. 2002). It can be easily visualized using immunocytochemistry and eliminates the issues surrounding incorporation periods, as well as the stress associated with injection procedures. Because staining for Ki-67 will identify all cycling cells, rather than only those currently in S-phase, counts of Ki-67-labeled cells will be higher (reports have found approximately doubled numbers) than BrdU-labeled cells in the same experimental situation. However, relative expression patterns of Ki-67 and BrdU incorporation have been found to mirror each other under a number of conditions, validating the use of either method according to convenience. It is common to study multiple stages of the neurogenic process in a single animal by injecting BrdU 3-4 weeks

prior to sacrifice to identify long-term cell survival, and also labeling Ki-67, DCX, nestin, or other markers to examine particular stages of proliferation and development.

Effects of neurogenesis on hippocampal function

Several possible functions have been proposed for this continuous production of new neurons. Computational network theories suggest that new neurons lend advantages in temporary storage and processing of new memories (Deisseroth, Singla et al. 2004). It has repeatedly been found that young granule cells show a reduced threshold to induction of long-term-potentiation (LTP) and lower sensitivity to GABAergic inhibition (Wang, Scott et al. 2000; Snyder, Kee et al. 2001). A large body of literature exists on the link between learning and neurogenesis, both positive and negative. Participation in learning tasks, particularly those involving hippocampal-dependent spatial memory formation (i.e., place recognition or maze tasks) or exposure to enriched or novel environments which facilitate spatial learning, are associated with increased hippocampal neurogenesis (Leuner, Gould et al. 2006). The most compelling arguments support a specific role in spatial pattern separation. Mice who are subjected to site-specific x-irradiation to ablate hippocampal cell proliferation develop significant impairment in the low spatial separation component of a challenging memory task, but function normally when presented with larger spatial separations and in other tasks of general spatial learning and memory (Clelland, Choi et al. 2009). Another research group found that the same irradiation treatment produced mice with normal spatial learning in the Morris water maze but impaired in the Barnes maze, which requires distinguishing a single escape tunnel from 40 possible locations and therefore uses more

fine spatial separation assessment than does the Morris maze (Raber, Rola et al. 2004). Interestingly, rats which primarily use a hippocampus-dependent place strategy in the Morris water maze exhibit lower rates of NPC proliferation than those which use a non-hippocampus-dependent cue strategy, although they do not differ in survival rates of newborn neurons (Epp and Galea 2009).

There is a compelling body of evidence that young granule cells are more responsive than their older counterparts to learning involving fear or anxiety. Survival and fos-activation of young granule cells are increased by exposure to a Morris water maze, but not in swim controls which are exposed to the stressful condition of the swim without the spatial learning component of hidden platforms (Gould, Beylin et al. 1999; Snyder, Radik et al. 2009). Additionally, inhibition of hippocampal neurogenesis using x-irradiation showed that the presence of immature neurons at the time of training is required for learning in the water maze (Snyder, Hong et al. 2005). Context-dependent fear conditioning also seems to be neurogenesis-dependent, as freezing is reduced in animals subject either to x-irradiation or to genetic ablation of neurogenesis, whereas cue-dependent fear conditioning and unstressed spatial learning are unaffected (Saxe, Battaglia et al. 2006). Specifically, the formation of a fear association during training, but not the unconditioned stressor or the expression of fear, has been found to significantly reduce proliferation of neurogenic cells of the dentate gyrus (Pham, McEwen et al. 2005).

Apart from those memory functions which have been specifically linked to new granule cells, it is likely that the continual alteration of hippocampal circuitry throughout

adulthood has gradual long-term effects on interactions with other brain regions which are intricately connected with the hippocampus. The limbic-hypothamalic-pituitary-adrenal (LHPA) axis encompasses inputs from the amygdala and prefrontal cortex (PFC) to the hippocampus, which, via the subiculum, inhibits the paraventricular nucleus (PVN) of the hypothalamus. In addition to this direct pathway between the hippocampus and PVN, an indirect pathway involves excitatory input from the hippocampus to the bed nucleus of the stria terminalis (BNST), which provides inhibition to the PVN. The parvocellular neurons of the PVN secrete corticotropin-releasing factor (CRF) onto the anterior lobe of the pituitary gland via the median eminence, resulting in the release of adrenocorticotropic releasing hormone (ACTH) into general circulation and ultimately stimulating production of glucocorticoids by the adrenal glands. In acute stress conditions, activity of this circuit and resulting production of glucocorticoids increase dramatically. Negative feedback regulation occurs via glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) at several points in the system, including direct inhibition upon CRF-secreting cells themselves, but largely through hippocampal cells (reviewed in (Lopez, Akil et al. 1999)).

The hippocampus is responsible for a general inhibitory tone on the PVN, as evidenced by hippocampal lesions that increase circulating glucocorticoid levels and prolong acute responses to stress (Fendler, Karmos et al. 1961). This inhibition is directly related to activation of local GR and MR, both of which are highly expressed in all regions of the hippocampus; as corticosterone binds with tenfold higher affinity to MR than to GR, MR is highly occupied at basal corticosterone levels, whereas GR occupation

comes into significant play only at higher stress levels (de Kloet, Oitzl et al. 1993). As such, local implants of corticosterone pellets or pharmacological blockades of GR flatten stress responses. Chronic stress conditions often result in lowered expression of both GR and MR, as well as a decrease in the MR/GR ratio, both of which contribute to impaired responsivity to elevated glucocorticoids. Antidepressant treatment returns receptor expression and function to normal (reviewed in (Lopez, Chalmers et al. 1998; Jankord and Herman 2008)). Additionally, CRF is produced and secreted within the hippocampus by a subset of GABAergic interneurons, and CRF_1 receptor is expressed by pyramidal cells, which have been shown to become activated under acute stress conditions (Chen, Brunson et al. 2004).

High glucocorticoid levels have been demonstrated to negatively influence the proliferation rate of hippocampal NPCs, as it is increased by adrenalectomy and decreased by acute corticosterone administration (Cameron and Gould 1994). However, NPCs do not themselves express either GR or MR (Cameron, Woolley et al. 1993), meaning this effect must be mediated by an indirect pathway. This pathway most likely involves NMDA-mediated excitatory signaling from the perforant path from the entorhinal cortex through the subiculum. NPC proliferation can be increased by lesion of this pathway or by administration of NMDA-receptor antagonists, either of which treatment will block the effect of corticosterone, and proliferation is decreased by direct injection of NMDA (Cameron, McEwen et al. 1995). Interestingly, NMDA blockade also increases rates of cell death of both mature and newborn granule cells (Gould, Cameron et al. 1994). Corticosterone levels have a U-shaped relationship with rates of granule cell

death. Complete removal of corticosterone via adrenalectomy results in cell loss, as do elevated levels commensurate with acute stress, meaning that low basal levels are optimal for survival. It is also notable that the age-related decreases in rates of both hippocampal cell proliferation and spontaneous neuronal death parallel the age-related increase in glucocorticoid secretion (Heine, Maslam et al. 2004).

Serotonin (5-HT) and the 5-HT_{1A} receptor in particular also play a large role in hippocampal response to stress. 5-HT_{1A} receptors are highly colocalized with GR and MR on GABAergic interneurons, which receive serotonergic innervations from the median and dorsal raphe. 5-HT release spikes in the presence of acute stress, therefore inhibiting the inhibitory influence of the hippocampus onto the PVN (Keeney, Jessop et al. 2006). Hippocampal 5-HT_{1A} expression and binding density are decreased by chronic stress; this decrease can be blocked by chronic antidepressant administration and appears to be mediated by glucocorticoids (Lopez, Chalmers et al. 1998). Simple regulation of 5-HT transmission does not appear to directly affect NPC proliferation, because neither acute 5-HT spikes nor depletion affect proliferation rate. However, treatment with a 5-HT_{2A} receptor antagonist has been reported to reduce proliferation acutely, and increase proliferation when given chronically; chronic administration of a 5-HT_{1A} receptor agonist increases proliferation rate (Santarelli, Saxe et al. 2003; Jha, Rajendran et al. 2006; Jha, Rajendran et al. 2008).

Role of neurotrophic factors

Neurotrophic factors, particularly brain-derived neurotrophic factor (BDNF), have been shown to play a role in the regulation of neurogenesis in the hippocampus.

Transgenic mice with disrupted BDNF signaling (either BDNF +/- or dominant-negative TrkB receptor) show increased proliferation but decreased 21-day survival rates, suggesting increased apoptosis during the survival period (Sairanen, Lucas et al. 2005). Because neurotrophins largely affect cell survival via inhibition of apoptotic pathways, this is a likely mechanism (Manji, Moore et al. 2000). Direct infusion of BDNF into the hippocampus dramatically increases the number of newborn neurons, even contralateral to the injection site, which suggests that this effect does not result exclusively from direct action upon NPCs or immature neurons (Scharfman, Goodman et al. 2005). However, evidence of a direct pathway has been demonstrated, in that both NPCs and immature neurons do express TrkB, and conditional deletion of the TrkB gene in these cells negatively affects their survival, axonal and dendritic growth, and ability to develop LTP (Bergami, Rimondini et al. 2008). In slice cultures, BDNF application enhances excitatory synaptic activity at CA1 pyramidal cells, offering a possible indirect pathway and tying BDNF-TrkB signaling to hippocampal learning and memory processes (Tyler and Pozzo-Miller 2001). Clinically, healthy subjects carrying the met allele of the val66met BDNF gene polymorphism have been found to have smaller hippocampal and parahippocampal volumes than those with the more common val/val genotype, providing further support for BDNF's importance in maintaining normal cell populations (Bueller, Aftab et al. 2006; Montag, Weber et al. 2009).

Another regulator of neurogenesis is vascular endothelial growth factor (VEGF). VEGF's receptor (Flk-1) is expressed on adult hippocampal NPCs (Yang, Zhang et al. 2003). Central VEGF infusion increases cell proliferation and increases numbers of

immature (DCX-expressing) neurons in the SGZ (Jin, Zhu et al. 2002; Warner-Schmidt and Duman 2007). Likewise, VEGF knockout mice show decreases in both cell proliferation and numbers of immature neurons (Sun, Jin et al. 2006), and viral-mediated VEGF overexpression increases proliferation and survival of new neurons, as well as improving performance in a Morris water maze and a passive avoidance learning task (Cao, Jiao et al. 2004). As is the case with BDNF, this evidence links VEGF to neurogenesis-associated memory enhancement without ruling out additional effects of non-neurogenic processes.

Stress and neurogenesis

Psychological stress has been found to impair many aspects of hippocampal neurogenesis: decreasing proliferation rate of NPCs, decreasing survival of neuroblasts and immature neurons, and decreasing growth and development of new neurons. (Although ischemic and oxidative stressors have also been found to negatively influence the neurogenic process, they will not be discussed here.)

Restraint or immobilization is a classic stressor which has been repeatedly demonstrated to inhibit proliferation, sometimes after only a single session but sometimes requiring repeated treatment, and also to reduce survival rate of new cells (Pham, Nacher et al. 2003; Vollmayr, Simonis et al. 2003; Duric and McCarson 2006; Koo and Duman 2008). Reduced hippcampal volume was produced by one experiment utilizing a chronic paradigm of prolonged (6 hours daily) restraint (Lee, Jarome et al. 2009). Chronic unpredictable stress or chronic mild stress paradigms, often considered to be more relevant to the pathogenesis of depression in humans, have also been used

to produce deficits in both proliferation and survival, although those including only mild stressors are somewhat less consistent (Heine, Zareno et al. 2005; Jayatissa, Bisgaard et al. 2006; Lee, Kim et al. 2006; Xu, Ku et al. 2007; Silva, Mesquita et al. 2008). A more naturally relevant stressor, the odor of a natural predator, also can reduce proliferation with only one exposure (Tanapat, Hastings et al. 2001).

Experiments involving social stress take advantage of male rats' propensity toward establishing dominance hierarchies within a colony to examine social subordination as a chronic low-intensity stress condition. The most elaborate social stress studies involve extensive burrow systems wherein animals can be monitored for home environment behavior, such that the effects of inhabiting various levels of a stable hierarchy can be compared between individual animals. In an experiment involving groups of four cohabitating rats, the dominant animal showed significantly higher numbers of surviving immature neurons than the subordinates, though the rate of NPC proliferation was unchanged (Kozorovitskiy and Gould 2004). A long-standing model using a colony of tree shrews, which form intense and long-lasting dominance hierarchies that are particularly stressful for the subordinate, shows dramatically decreased proliferation, dramatically increased adrenal weights, and decreased hippocampal volume (Lucassen, Vollmann-Honsdorf et al. 2001; Simon, Czeh et al. 2005). The social defeat model (also called the resident-intruder model) restricts psychosocial stress into discrete episodes of conflict: the experimental animal (called the intruder) is only exposed to a larger dominant animal in its home territory (the resident) for short sessions, which can be performed on acute or chronic timelines. In

some variations, the intruder is left in visual and olfactory contact with the resident (e.g., separated by a mesh barrier) for a period extending beyond the physical interaction. Chronic daily sessions have been shown to reduce both cell proliferation and neuronal survival (Czeh, Welt et al. 2002), while a single session reduces short- and long-term survival, but not proliferation (Thomas, Hotsenpiller et al. 2007). Additionally, social isolation has been shown to intensify deleterious effects of other stressors (Stranahan, Khalil et al. 2006).

Studies involving electric footshock have attempted to tease apart the importance of stress-related factors such as fear conditioning and behavioral control. A single session of footshock, either inescapable or as part of avoidance conditioning, has been in some cases enough to reduce NPC proliferation by 50%, regardless of whether animals develop learned helplessness behavior, suggesting that any stressor can inhibit neurogenesis when administered at sufficient intensity and duration (Malberg and Duman 2003; Vollmayr, Simonis et al. 2003). However, a report involving tailshock found that a single session reduced proliferation only in those rats developing learned helplessness (Chen, Pandey et al. 2006). Studies of yoked pairs, in which one animal controls the duration of shock for both partners, have separately found that both escapable and inescapable conditions result in decreased proliferation, that only the inescapable condition does so, or that neither has any effect on proliferation (Westenbroek, Den Boer et al. 2004; Bland, Schmid et al. 2006; Shors, Mathew et al. 2007). Further complicating the story is the fact that virtually all experiments in this area are performed on male animals only; when females are included they often show very

different results, such as increased proliferation when males demonstrate a decrease, or no effect where males show a large one (Westenbroek, Den Boer et al. 2004; Shors, Mathew et al. 2007).

As mentioned above, a particularly interesting series of experiments discovered that proliferation was only decreased by inescapable shock when the protocol timeline allowed contextual fear associations to occur, suggesting strongly that the key factor is not stress or fear itself, but the emotionally charged learning that takes place under stressful conditions (Pham, McEwen et al. 2005). However, another study using a similar active avoidance protocol that should allow for contextual conditioning found no change in proliferation or survival rates, even when substantial learning had occurred (Van der Borght, Meerlo et al. 2005).

Stressful experiences in prenatal and early life, which have lasting effects on behavioral stress response into adulthood, have similarly lasting effects on both basal neurogenesis and its response to stress. Maternally separated rats show decreased NPC proliferation and immature neuron survival, but do not show normal stress-related suppression of these (Mirescu, Peters et al. 2004). Likewise, adult offspring of dams subjected to restraint stress during gestation show suppressed proliferation and survival, which can be rescued to normal levels by daily handling during the early postnatal period (Lemaire, Lamarque et al. 2006).

Antidepressants and neurogenesis

A great number of antidepressant drugs have been shown to increase various aspects of neurogenesis. NPC proliferation rate and immature neuron survival rate have

been upregulated by fourteen or more days of treatment with the selective serotonin reuptake inhibitor (SSRI) fluoxetine, but not by shorter regimens (Malberg, Eisch et al. 2000; Kodama, Fujioka et al. 2004; Huang and Herbert 2006; Marcussen, Flagstad et al. 2008), although this effect is not universally observed (Cowen, Takase et al. 2008; David, Samuels et al. 2009). Experiments involving the SSRIs citalopram and escitalopram have been less successful, with negative results outnumbering positive ones regarding both proliferation and survival (Jaako-Movits, Zharkovsky et al. 2006; Jayatissa, Bisgaard et al. 2006). The tricyclic antidepressant imipramine, although less frequently used in studies of neurogenesis, has been reported to positively impact both proliferation and survival (Keilhoff, Becker et al. 2006; Surget, Saxe et al. 2008). Additionally, other classes of drugs which are not clinically approved but have shown antidepressant properties in laboratory animals, such as CRF_1 antagonists and $V1_b$ antagonists, have been reported to similarly rescue proliferation rate from a stress-induced deficit (Alonso, Griebel et al. 2004; Surget, Saxe et al. 2008). Mood stabilizers lithium and valproate have shown robust effects in enhancing both proliferation and survival (Chen, Rajkowska et al. 2000; Hao, Creson et al. 2004; Silva, Mesquita et al. 2008), whereas atypical antipsychotics such as olanzapine and clozapine have shown inconsistent results, sometimes positively affecting proliferation, but never changing numbers of surviving cells (Halim, Weickert et al. 2004; Kodama, Fujioka et al. 2004).

Non-drug treatments which have behavioral antidepressant effects also positively affect neurogenesis. A single session of electroconvulsive seizure (ECS), which is analogous to the electroconvulsant therapy used clinically to treat severe cases of

major depression, causes a profound increase in the number of new neurons surviving up to three months afterward. With multiple sessions, NPC proliferation rate also shows a dramatic increase, greater than that seen with fluoxetine (Madsen, Treschow et al. 2000; Malberg, Eisch et al. 2000). ECS has even been able to restore some degree of neurogenesis after disruption by x-irradiation, as well as restoring the corresponding deficits in contextual fear conditioning (Warner-Schmidt, Madsen et al. 2008). A home cage environment enriched to encourage exploratory behavior, as discussed above, promotes neurogenesis in a spatial learning context, and also relieves depressive and anxious behaviors under conditions of stress (Veena, Srikumar et al. 2009). Exercise, either forced or voluntary, likewise has been shown to both increase NPC proliferation and decrease depressive and anxious behaviors (Olson, Eadie et al. 2006; Yi, Hwang et al. 2009).

The neurogenesis hypothesis of depression

Based on the evidence described above that hippocampal neurogenesis is reduced under stressful conditions, such as those leading laboratory animals to develop behavioral depression, and is increased by antidepressant drugs and other antidepressant treatments, a hypothesis has emerged that neurogenesis and other related aspects of hippocampal plasticity are integrally involved in the pathophysiology of major depressive disorder (MDD) and its effective treatment. Some experiments have even suggested that neurogenesis is necessary for the therapeutic effects of antidepressants (Santarelli, Saxe et al. 2003). Clinical evidence supporting this hypothesis includes the persistent finding of reduced hippocampal volume in MRI or

postmortem studies of MDD patients. Meta-analysis of 32 publications has found that volume is significantly reduced with greater than one lifetime major depressive episode or greater than two years of illness, suggesting that the observed atrophy is resultant from the burden of illness rather than being a preexisting risk factor (McKinnon, Yucel et al. 2009). Additionally, some evidence has been found for increased proliferation of NPCs in antidepressant-treated vs. untreated MDD (Boldrini, Underwood et al. 2009).

However, the hypothesis has been recently subjected to serious criticisms. Many factors other than decreased neuron number could account for hippocampal volume changes: tissue from MDD patients has been found to have increased granule cell and pyramidal cell density, presumably from decreased neuropil, and the possibility of altered fluid content also exists (Stockmeier, Mahajan et al. 2004). The effects of chronic stress or experimentally elevated corticosterone concentrations on dendritic atrophy and loss of synapses have been well documented in laboratory animals (Sousa, Lukoyanov et al. 2000; Vyas, Mitra et al. 2002; Tata and Anderson 2009). This atrophy can be reversed by administration of antidepressants of multiple classes, and in fact, the behavioral effects of chronic stress and antidepressants in the sucrose consumption test and forced swim test have been found to be associated more closely with the complexity of the dendritic arbor of granule and pyramidal cells than with neurogenesis (Bessa, Ferreira et al. 2009).

Another strike against the hypothesis is the finding that experimental disruption of neurogenesis does not produce depression-like behavior, nor does it make animals more sensitive to the behavioral effects of chronic stress (Surget, Saxe et al. 2008;

Jayatissa, Henningsen et al. 2009). Additionally, some reports of antidepressants at behaviorally active doses show no action upon NPC proliferation or immature neuron number, as detailed above. Another complication arises when comparing studies using x-irradiation to block neurogenesis and those using the chemical agent methylazoxymethanol (MAM): these show quite different effects upon antidepressant actions (cf. (Surget, Saxe et al. 2008; Bessa, Ferreira et al. 2009), making it likely that some of the effects of hippocampal x-irradiation are due less to deficits in neurogenesis than to associated alterations in the neurotrophic environment.

Project rationale

At its inception, this project was intended to augment the existing understanding of the regulation of adult hippocampal neurogenesis by exploring interactions with the CRF system. The starting hypothesis was that, if antidepressant treatments have a positive effect on neurogenic processes (either at basal levels or as rescue from the negative effects of stress), and CRF_1 antagonists have reliable antidepressant effects, then these drugs should similarly upregulate neurogenesis. If correct, this hypothesis would support the importance of hippocampal neurogenesis as a unifying point in the pathophysiology of depression and the efficacy of antidepressant mechanisms, rather than a mere artifact of monoaminergic manipulation. However, the difficulties encountered in confirming and extending the findings in the existing literature on the effects of stress and monoaminergic antidepressants led the project in quite a different direction: namely, a larger exploration of the complexities and shortfalls of the existing neurogenesis hypothesis of depression.

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2: Effects of stress

The initial objective of this project was to establish a stress paradigm which would reliably and robustly decrease the rate of neural progenitor cell (NPC) proliferation in the dentate gyrus in adult animals, by direct replication of stressors reported as successful in the literature as detailed in the section "Stress and Neurogenesis" in the previous chapter. The stressors explored were restraint (modeled after the report of (Vollmayr, Simonis et al. 2003)), tailshock (modeled after reports of (Malberg and Duman 2003; Vollmayr, Simonis et al. 2003)), and social defeat (modeled after reports of (Yap, Takase et al. 2006; Thomas, Hotsenpiller et al. 2007)), in chronological order. Additionally, one experiment included animals from a selectively bred stress-sensitive "hyper" line (Weiss, West et al. 2008)). As young adults, rats of this line react to tail shock stress by developing hyperactive locomotion for a short period of time, but later in life (such as the ten month old animals used in this experiment) they respond to stress with a behavioral depression that is much longer-lasting than that seen in non-selected rats (approximately four weeks rather than one week).

Although NPC proliferation rate was the major target of interest, assessment of other neurogenesis-related factors was made in many of these experiments. The effect of social defeat stress on rates of short-term and long-term survival of immature neurons was measured, as well as the effect on glucocorticoid receptor (GR) expression. Effects of several of the stressors on brain-derived neurotrophic factor (BDNF) expression were also measured.

Materials and methods

Animals

Adult male Sprague-Dawley rats (Charles River, Wilmington MA) were obtained one week prior to the starts of the experimental protocols. To partially control for age, animals in acute studies were approximately ten weeks old and animals in chronic studies were approximately eight weeks old at the start, so that ages would be comparable at the time of sacrifice. The off-site tail shock experiment was an exception to this: animals in this experiment were male Sprague-Dawley rats, approximately ten months old, chosen from an in-house breeding colony originally established using lines from Charles River, including animals from the selectively bred HYPER line and from a non-selected line. Resident animals for the social defeat procedures were male Long-Evans retired breeders (Charles River), pair-housed with sexually mature females.

In all cases, animals were maintained in standard laboratory conditions: pair-housed (except as noted for social defeat cohorts) in polycarbonate cages (40 x 28 x 20 cm for social defeat residents; 30 x 20 x 20 cm for all others) with corn cob bedding, on a 12:12 light cycle, with *ad libitum* access to food and water. All procedures used were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University and are in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

Monitor hyper rating

Several months prior to their inclusion in the stress experiment described here, animals of the HYPER line were exposed to a single tailshock session identical to the one used in this experiment. Following this, they were individually housed in cages equipped with a locomotion tracking system for approximately ten days. Their nocturnal activity was analyzed by number of beam-break counts. They were classified as "great" or "good" according to the magnitude of their hyperactive response to the stressor, or "poor" if no or very little hyperactivity was seen.

Stress procedures

Restraint stress

Between 10:00 and 12:00 am animals in the restraint group were transported to another room, removed from their cages, and restrained in flexible plastic DecapiCones (Braintree Scientific, Braintree MA) for two hours. During this time, control animals remained in their home cages.

Tailshock stress

Tailshock experiments were conducted under two different protocols with different sets of equipment in different locations. Although both were performed on the Emory University campus, for ease of reading one will be referred to as "on-site" and the other as "off-site".

In the on-site experiments, between 10:00 and 12:00 am animals in the tailshock group were transported to another room, removed from their cages, and restrained in individual wire mesh cylinders. Two electrodes were taped to either side of the tails of

four animals concurrently and connected in series to ensure that each animal received an equal current. Each tailshock session consisted of 60 1.0 mA shocks, delivered on a variable interval schedule averaging one shock per minute, for a one hour period. For those animals subjected to repeated sessions, these took place on consecutive days. Blood was collected via tail nick 20 minutes from the start of the session and again at the conclusion of the session. At the end of each session, the animals were returned to their home cages. During this time, control animals remained in their home cages. Tail blood was also collected from these animals at the same time of day, but each control animal was only subjected to this procedure on one of the three days in order to minimize stress.

In the off-site experiments, between 10:00 and 12:00 am animals in the tailshock group were transported to another room, removed from their cages, and placed into plexiglass containers. Two electrodes were taped to either side of the tails of eight animals concurrently and connected in series to ensure that each animal received an equal current. The tailshock session lasted for three hours, within which shocks gradually increased from 1.0 to 2.5 mA, delivered on a variable interval schedule averaging one shock per minute. During this time, control animals remained in their home cages.

Social defeat stress

Animals subjected to social defeat were housed individually, in order to maximize the impact of the defeat stress by removing the soothing effects of non-

hostile conspecifics and to eliminate the additional variable of social dominance or subordinance in the home cage pair. Control animals were housed in pairs.

Several weeks in advance of the social defeat experiment, resident animals were trained for aggressive territorial behavior by challenging them multiple times with smaller intruder animals in the social defeat procedure detailed below. Only those animals which met criteria for sufficiently aggressive and dominant behaviors were used as residents in the social defeat experiments. These criteria included minimum latency to first attack and minimum latency to pin.

Between 2:00 and 4:00 pm, resident animals were transported to another room, where the female cagemates were removed, and were left undisturbed for one hour. Experimental animals in the defeat group were then transported from the colony and each one placed into the homecage of a resident, separated by a wire mesh barrier which allowed visual and olfactory contact only for the first 2 minutes of the encounter. The barrier was then removed and the animals allowed to interact for 20 minutes. If the intruder was injured during the encounter, the barrier was replaced for the remainder of the session. For those animals subjected to repeated sessions, these took place on Mondays, Wednesdays, and Fridays, with resident-intruder pairs systematically shuffled for each session to equalize the defeat experience between experimental animals. Behavior was monitored during these sessions, and any intruder which did not display sufficient defeated behavior was omitted from the experimental analysis.

Serum corticosterone analysis

Blood samples obtained from the on-site tailshock experiment were centrifuged to obtain serum, which was analyzed via radioimmunoassay for corticosterone levels. A 100 µl aliquot of 1:200 rat serum in assay buffer (MP Biomedicals, Solon OH) was incubated with 200 µl of highly specific antibody and 200 µl [^{125}I]-corticosterone at room temperature for 2 hours. The bound complexes were separated via addition of 500 µl of secondary antibody, followed by centrifugation at 2500 x g for 25 minutes, and the unbound complexes were decanted to waste. The residual pellets were counted for 3 minutes in a LKB Clin-Gamma gamma counter. Quantification was achieved via log/logit data reduction.

BrdU administration and sacrifice

BrdU (200 mg/kg in 0.9% saline) was administered via a single *ip* injection according to the timelines given for each experiment. For assessment of cell proliferation rate, BrdU injection was given immediately following the final stress session and animals were sacrificed 24 hours later; for assessment of newborn cell survival, BrdU injection was given before the first stress session and animals were sacrificed one (short-term survival) or three (long-term survival) weeks later. At the time of sacrifice, animals were deeply anesthesized with pentobarbital and transcardially perfused with cold 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde. The brains were removed, post-fixed overnight, and equilibrated in a 30% sucrose solution. Sections of 25 µm through the entire hippocampus (bregma –2.4 to –6.2) were cut on a cryostat and mounted on slides for ICC and *in situ* hybridization procedures.

Immunocytochemistry

Immunocytochemical staining was performed on every twelfth section throughout the hippocampus. For BrdU staining, antigen retrieval was performed by incubation in 50% formamide/SSC at 65° C followed by 2M HCl at 37° C, then neutralization in 0.1 M boric acid (pH 8.5). After quenching in 2% hydrogen peroxide, they were blocked in 3% normal horse serum (Vector, Burlingame, CA) and incubated overnight in 1:100 mouse monoclonal anti-BrdU antibody (Becton-Dickinson, Franklin Lakes, NJ). The next day, they were treated with 1:200 horse anti-mouse rat-adsorbed secondary antibody, then treated with a Vectastain ABC kit and visualized with DAB substrate (all Vector).

Stained sections were examined at 40x magnification under a light microscope. Labeled cells were counted if they were within the subgranular zone of the dentate gyrus or within one cell width from its edge. A total number of positively labeled cells was acquired for each animal and multiplied by 12 to approximate the number of labeled cells throughout the entire dentate gyrus. Additionally, cell counts were divided into dorsal and ventral parts of the dentate gyrus. This procedure follows established guidelines for unbiased rare event stereology (Mouton 2002). All cell counting was performed by the same individual under treatment-blind conditions.

A Student's t-test or one-way ANOVA (depending on whether multiple levels of stress were included in each experiment) was performed on each of the resulting measures, with total dentate gyrus and dorsal and ventral regions considered

separately. The criterion for significance was $p < .05$. If justified by ANOVA, post hoc comparisons were made between individual groups and control using Dunnett's test.

In situ hybridization

mRNA was quantified using *in situ* hybridization with an ^{35}S-labeled riboprobe. As in the immunocytological protocol above, every twelfth section was treated and analyzed. BDNF and GR riboprobes were constructed from cDNA inserts including the full coding regions plus some flanking sequence, ligated into pR1112-8 (BDNF) or pGemA (GR) plasmids. Radiolabeled antisense cRNAs were synthesized by incorporating [^{35}S]-CTP into the probes. The transcription reactions were performed utilizing the Ambion MAXIscript kit with T3 RNA polymerase for BDNF or T7 RNA polymerase for GR, according to the instructions provided. Following transcription and removal of the cDNA template with DNase, the cRNA probes were recovered through gel filtration using a G-50 Sephadex Quick Spin column.

The slides underwent 10 minutes of acetylation (0.5% acetic anhydride in 0.1 mol/L triethanolamine, pH 8.0), two rinses in 2X SSC, and dehydration through a graded ethanol series. The sections were then air-dried for at least one hour prior to hybridization. The brain sections were hybridized overnight at 60°C with 1-2 x 10^6 cpm of [^{35}S]-labeled cRNA probe diluted into hybridization buffer (50% formamide, 10% dextran sulfate, 0.3 M NaCl, 1X Denhardt's solution, 10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mg/ml yeast tRNA, 10 mM dithiothreitol [DTT]) in humidified Nunc trays.

The next day, slides were allowed to cool to room temperature before being washed four times in 4X saline-sodium citrate buffer (SSC). The sections were then treated with 250 µg/ml RNase A for 30 minutes at 37°C. Subsequently, the slides underwent a series of SSC washes (supplemented with 1 mM DTT) with salt concentrations decreasing from 2X to 0.5X, followed by a 60 minute high stringency wash with 0.1X SSC + 1mM DTT at 60°C, then dehydration through a graded ethanol series. The slides were air-dried and then apposed to Kodak Biomax MR film until signal reached the desired intensity.

Images on film were digitized with CCD-72 image analysis system equipped with a camera. Semiquantitative analysis was performed using AIS software. Messenger RNA expression levels were calculated by subtracting the neutral background density from the specific signal. Sections were matched for rostrocaudal level and analyzed for values representing the regions CA1/2, CA3, and dentate gyrus, which were then averaged between sections to produce a single value for each region for each animal.

A Student's t-test or one-way ANOVA (depending on whether multiple levels of stress were included in each experiment) was performed on each of the resulting measures, with total dentate gyrus and dorsal and ventral regions considered separately. The criterion for significance was $p < .05$. If justified by ANOVA, post hoc comparisons were made between individual groups and control using Dunnett's test.

Results

Acute restraint stress

A single two hour session of restraint stress had no significant effect on cell proliferation rate, either in the entire dentate gyrus or in the dorsal or ventral parts. (Figure 2-1; n=8 per group.)

Tail shock stress

The on-site stress procedure was verified by measurements of serum corticosterone taken twice during each stress session (Figure 2-2.). Elevated levels (over 40 ng/ml) at the 20 minute timepoint show the intensity of the stressor, elevated levels at the 60 minute timepoint show that this stress intensity was maintained throughout the entire session, and elevated levels during the second and third sessions show that animals did not appreciably habituate to the tail shock stress over the course of the experiment. Very low levels in the control group (less than 50 ng/ml, with the exception of one animal at the session 1 – 60 minute timepoint) establish a minimally stressful baseline housing condition and show that the tail nick procedure itself does not result in elevated corticosterone.

As the off-site tail shock procedure has been previously validated as reliably producing behavioral depression, no additional verification of the stress level of the protocol was performed (Weiss, Bailey et al. 1980; Simson, Weiss et al. 1986).

No significant effect on cell proliferation was seen of either the on-site procedure, consisting of one daily one-hour session performed on each of three

consecutive days, or the off-site procedure, consisting of a single three-hour session (Figures 2-3 and 2-4; on-site n=12 per group; off-site n=8 per group). It should be noted that the animals used in the off-site procedure were aged approximately ten months, in contrast to the standard "young adult" age of ten weeks used in the other experiments. The baseline (non-stressed) rate of cell proliferation is dramatically lower, consistent with reports of decreased neurogenesis in aged animals (Kuhn, Dickinson-Anson et al. 1996).

No significant effect of either tail shock procedure was seen on BDNF mRNA expression in the CA1/2, CA3, or dentate gyrus (Figures 2-5 and 2-6; on-site n=12 per group; off-site n=8 per group).

Tail shock stress in HYPER line

A cohort of animals from the selectively bred HYPER line was included in the off-site tail shock experiment. These showed no effect of the stress procedure on cell proliferation rate (Figure 2-7; n=7 for the tail shock group; n=8 for the control group). However, post-experimental analysis of the effect of monitor hyper rating showed that control animals rated "great" or "good" had a significantly lower proliferation rate than control animals rated "poor" (Figure 2-7; p=0.0004; n=4 per group). The proliferation rate of "poor" HYPER animals was similar to that of non-selected animals.

No significant effect of the tail shock procedure or of monitor hyper rating was seen on BDNF mRNA expression in any area examined (Figure 2-8; N= 4 for the

"great/good" control group; n=7 for the "great/good" tail shock group; n=4 for the "poor" control group).

Social defeat stress

No significant effect on cell proliferation rate was seen after either one day (one session), 7 days (3 sessions), or 21 days (9 sessions) of social defeat stress, in either in the entire dentate gyrus or in the dorsal or ventral parts (Figure 2-9; n =6 for each social defeat group; n=8 for control group). Neither was there any effect of social defeat during a short (7 days) or long (21 days) survival period (Figures 2-10 and 2-11; n=12 per group).

Expression of BDNF mRNA was unchanged after exposure to a single social defeat session (Figure 2-12). Expression of GR mRNA was unchanged after exposure to one day (one session), 7 days (3 sessions), or 21 days (9 sessions) of social defeat (Figure 2-13).

Discussion

The complete inability to induce a decrease in NPC proliferation in the dentate gyrus of normal adult rats with any of three different stressors was quite unexpected. Given that a great variety of stressors have been reported to produce substantial neurogenesis deficits, so long as the stress intensity was sufficient, it is very surprising that even by increasing the severity and length of exposure of the tail shock and social defeat protocols to the upper boundaries of acceptable laboratory conditions, no degree of the expected result was observed.

Because the original goal of this project was to explore a particular system's relation to the regulation of neurogenesis under both stressed and unstressed conditions, rather than undertaking a comprehensive study of the relation between stress and neurogenesis, the experiments presented in this chapter were not designed *de novo* but were intended as replications of specific successful experiments reported in the literature. The mildest stressor, a single period of restraint was modeled after a successful reduction of cell proliferation of approximately 30% reported by (Vollmayr, Simonis et al. 2003). After failure to confirm this result, tail shock was chosen as the most intense stressor available and familiar to this lab. The first tail shock experiment (on-site) was performed multiple times, initially according to the single session footshock protocols reported as successful by (Malberg and Duman 2003; Vollmayr, Simonis et al. 2003) and, after initial failure to produce a result, using increasing shock current over multiple sessions to no better results (only the final, most intense, version of the experiment is included in the Results section). Even precise application of a long-standing tail shock procedure with behavioral and endocrine validation (the off-site experiment) yielded no success. Finally, social defeat stress, thought by some to be a more salient and naturalistic stressor, and therefore more relevant to the development of models mimicking human depression, was explored in both acute and chronic versions, according to the models of (Yap, Takase et al. 2006; Pulliam, Dawaghreh et al. 2009). After suggestions that survival of neuroblasts through maturation might be affected by the stressor while cell proliferation was not, as reported by (Thomas, Hotsenpiller et al. 2007), alternate experimental timelines were attempted, assessing

survival of BrdU-labeled cells over one or three weeks of continued defeat sessions, with again no stress effect seen.

The present results demonstrate that a severe stressor is not sufficient to alter the proliferation rate of neural progenitor cells, which is discordant with the commonly accepted hypothesis that hippocampal neurogenesis is consistently regulated by stress. It would seem that there is an as yet unidentified component of stress or some factor concurrent with it which is responsible for the effects of "stress" on neurogenesis, rather than the stress *per se*. A number of possible speculative factors include: subtle characteristics of the stress experiences, such as accessible coping mechanisms, audible or visible cues, or handling; differences in housing environments such as ambient noise level or staff activity; prior experiences in breeding facilities or shipping; etc. Because each different stress procedure was conducted in a different room and animals for the off-site tail shock experiment were housed in a different colony, it is unlikely that some condition unique to the environment is responsible for the lack of effects seen. Additionally, because this lab specializes in stress and behavior, the personnel responsible for animal contact are well experienced in minimizing stressful confounds in housing and experimental conditions, and other (non-neurogenesis-related) effects of stress were observed in other experiments concurrently using the same facilities and animal suppliers. Although differences in levels of neurogenesis between rat strains do exist (Husum, Aznar et al. 2006; Kronenberg, Lippoldt et al. 2007; Alahmed and Herbert 2008), the strain used in these experiments (Sprague-Dawley) is by far the most commonly utilized in the neurogenesis literature and is used in the particular

experiments cited as examples for the stress procedures performed here, so strain differences are not a consideration in the failure to replicate.

It is also possible that individual variation, specifically a predisposition to depression as a result of hereditary factors or of early life experience, plays an important role in stress-induced decreases in neurogenesis. There is some support for this hypothesis, as altered neurogenesis has been found in the Flinders sensitive rat, a selectively bred line with some characteristics of depression (Bjornebekk, Mathe et al. 2005; Husum, Aznar et al. 2006), and in adult rats subjected to maternal separation as pups (Mirescu, Peters et al. 2004; Karten, Olariu et al. 2005). The HYPER line was used here in the hope that these animals' enhanced behavioral responsiveness to stress might be linked to an enhanced response of neurogenesis to stress. This did not turn out to be the case, as HYPER rats, like their non-selected counterparts, showed no effect of tail shock on either cell proliferation or hippocampal BDNF expression, but analysis of those with high monitor hyper ratings (which can be considered "true" hypers as the hyper phenotype is not fully penetrant without continued selective breeding in every generation) did show a significantly lower rate of baseline (unstressed) proliferation. This finding (altered baseline neurogenesis without altered stress responsivity) is similar to reports of other rat models of depression, such as the Flinders sensitive line and early life stress experience mentioned above.

Another possibility, discussed in the introductory chapter, is that the experience of stress has been conflated in many experiments with the acquisition of fear learning.

Some reports allude to this by assessing the development of learned helplessness following a stressor. (Vollmayr, Simonis et al. 2003) reported that only those animals which became helpless following an inescapable shock showed decreased cell proliferation, while those which were exposed to the shock but did not become helpless maintained normal proliferation levels. (Malberg and Duman 2003) eliminated animals which failed a post-shock test criterion establishing helplessness from their cohorts, such that their results comprise not strictly an effect of inescapable shock but of learned helplessness, in concordance with (Vollmayr, Simonis et al. 2003). This hypothesis is supported by results from (Pham, McEwen et al. 2005), who reported a shock-induced decrease in proliferation only when animals had previous exposure to the shock chamber, allowing them to develop normal fear conditioning. Animals which were shocked immediately upon introduction to the chamber did not develop fear conditioning and did not exhibit decreased cell proliferation, despite highly elevated corticosterone levels.

The lack of effect of these stress procedures on BDNF mRNA expression was likewise surprising. Although BDNF has sometimes been shown to *increase* immediately following acute stress, a detailed study of the time-course of expression revealed that it reaches a nadir 24 hours post-stress (Marmigere, Givalois et al. 2003; Pizarro, Lumley et al. 2004). Other studies of repeated stress show BDNF expression is lowest immediately following the final stress, but still significantly lower than control 24 hours post-stress (Murakami, Imbe et al. 2005; Bland, Tamlyn et al. 2007). Based on this, 24 hours is an appropriate time point at which we would expect to see decreased BDNF mRNA

expression following an acute stressor. However, the strong link between BDNF expression and regulation of neurogenesis (as described by evidence in the introductory chapter) makes it perhaps less surprising to see no change in BDNF expression than it would be to see a change in BDNF in the absence of a change in patterns of neurogenesis.

Hippocampal expression of GR mRNA and protein is often reported to be slightly decreased after stress, under both acute and chronic conditions (Chen, Tang et al. 2008; Zhou, Li et al. 2008; Adzic, Djordjevic et al. 2009). However, contrasting reports suggest that only acute stress produces an appreciable reduction and that expression normalizes with repeated stress exposures (Aguilar-Valles, Sanchez et al. 2005; Romeo, Ali et al. 2008). Thus, the lack of change in GR expression as a result of social defeat stress is not inconsistent with the literature.

In summary, the results presented in this chapter challenge the prevalent view that adult hippocampal neurogenesis, including the rate of NPC proliferation, is negatively regulated by any stress of sufficient intensity. The procedures used cover a range of stress modalities and are inarguably profoundly stressful, yet they failed to alter any neurogenic measure assessed here. The complex nature of stress makes reproducibility of results a common problem in the field, because it is impossible to monitor and report every variable contributing to stress level in an animal, but these experiments have credibly negated such subtle environmental factors by varying procedures, locations, and even animal populations, while consistently producing very

intense levels of stress in the experimental groups and minimal levels in the controls. The simple “stress decreases neurogenesis” paradigm has been challenged by several other hypotheses, as presented briefly above, and these findings strongly support one of these more complex explanations.

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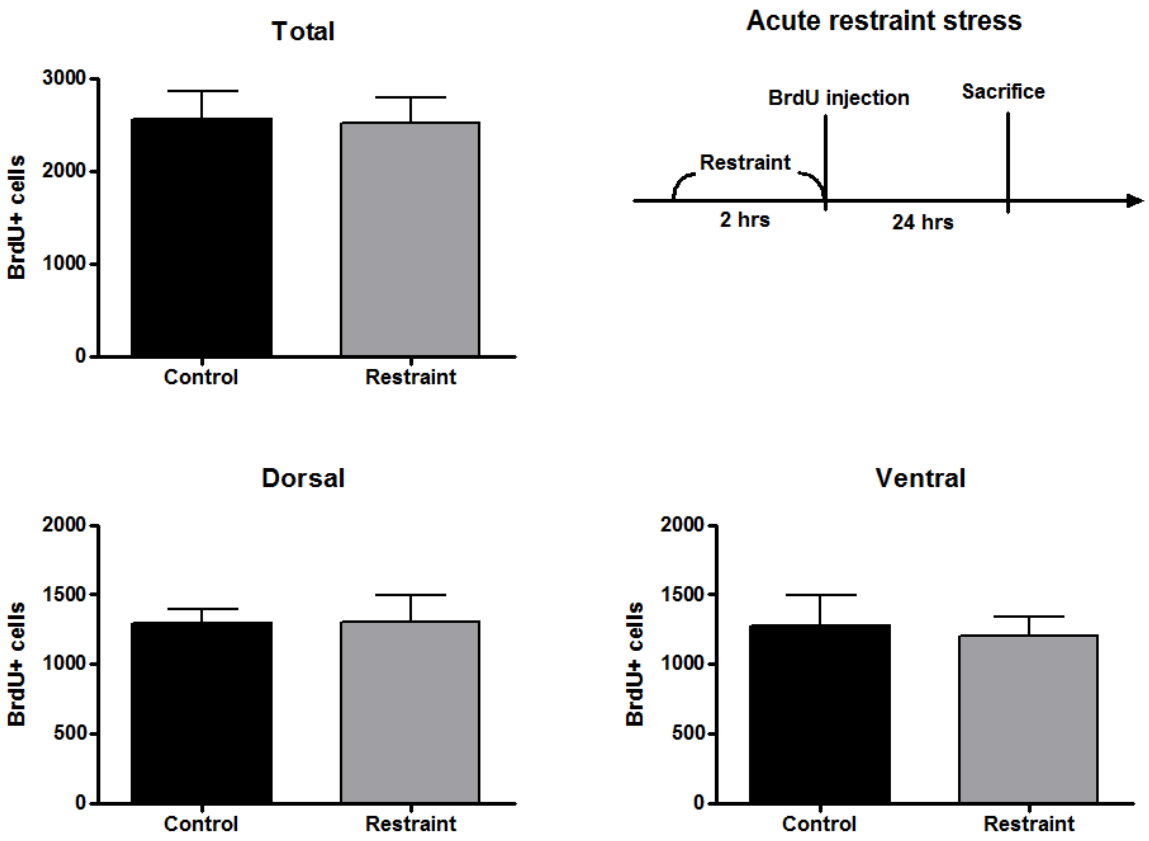

Figure 2-1. BrdU cell counts indicating cell proliferation rate in the dentate gyrus. Data are presented from the entire hippocampus and from the dorsal and ventral regions separated. T-test was not significant for any region. N= 8 per group.

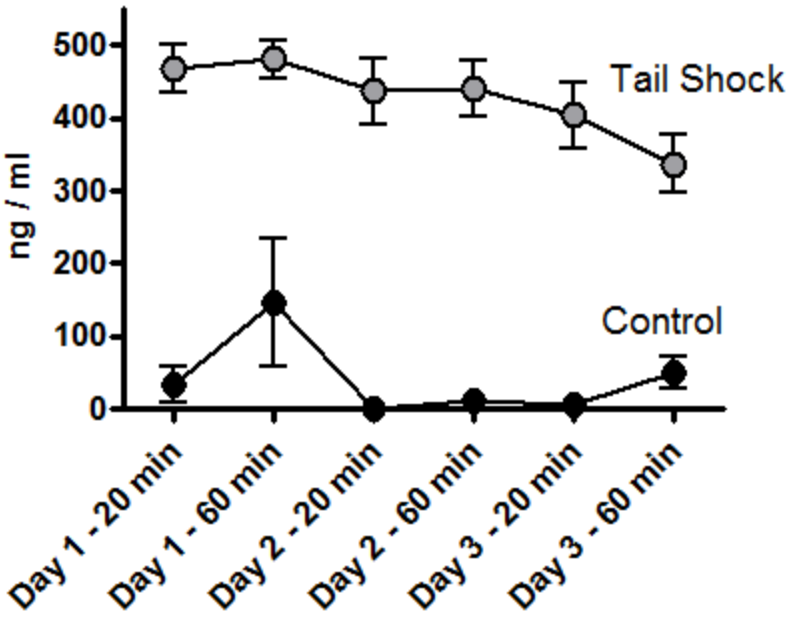

Figure 2-2. Serum corticosterone levels during the on-site tail shock experiment. N=8 for tail shock group; n=2-4 at each timepoint for control group.

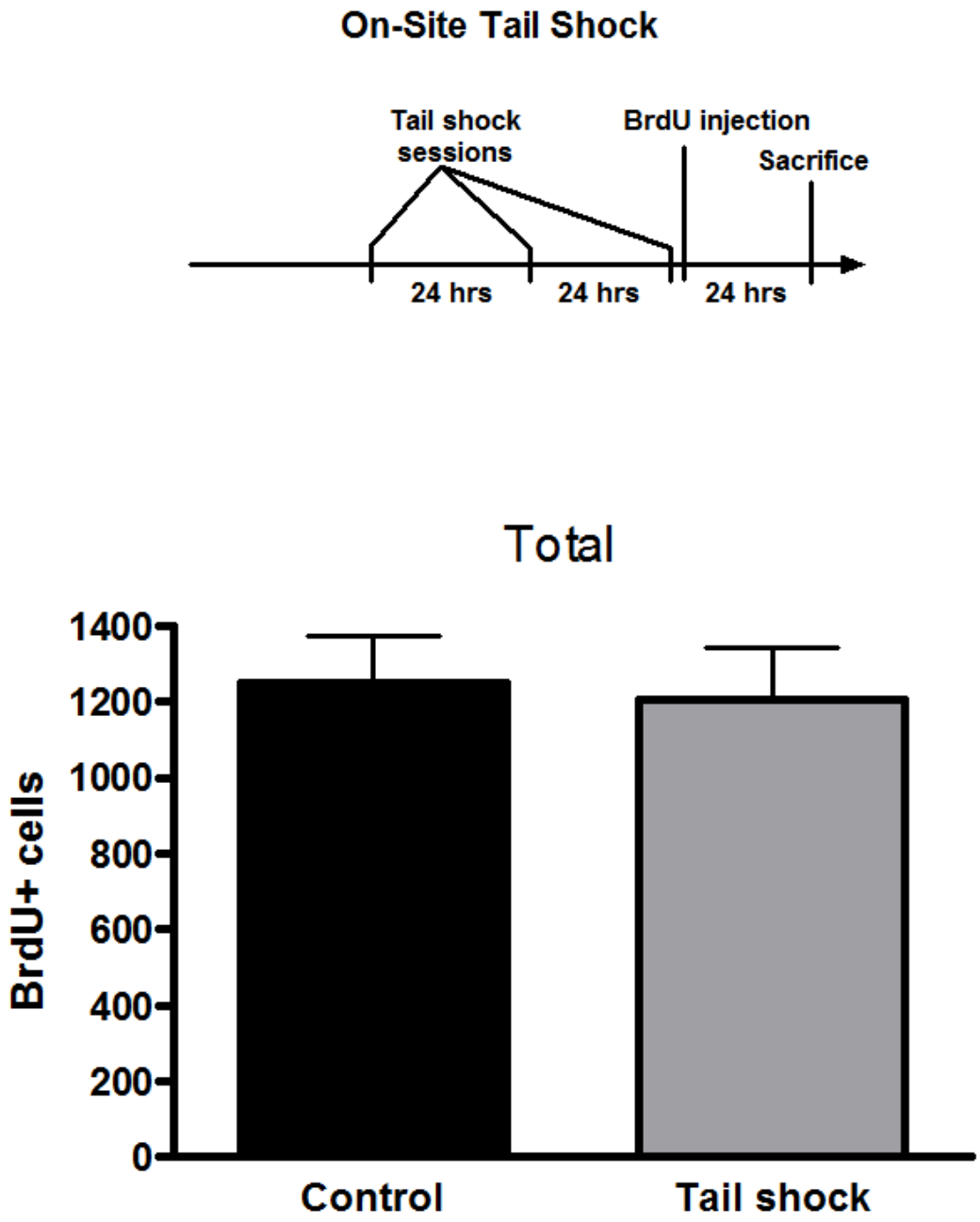

Figure 2-3. BrdU cell counts indicating cell proliferation rate in the dentate gyrus. Data are presented from the entire hippocampus. T-test was not significant. N= 12 per group.

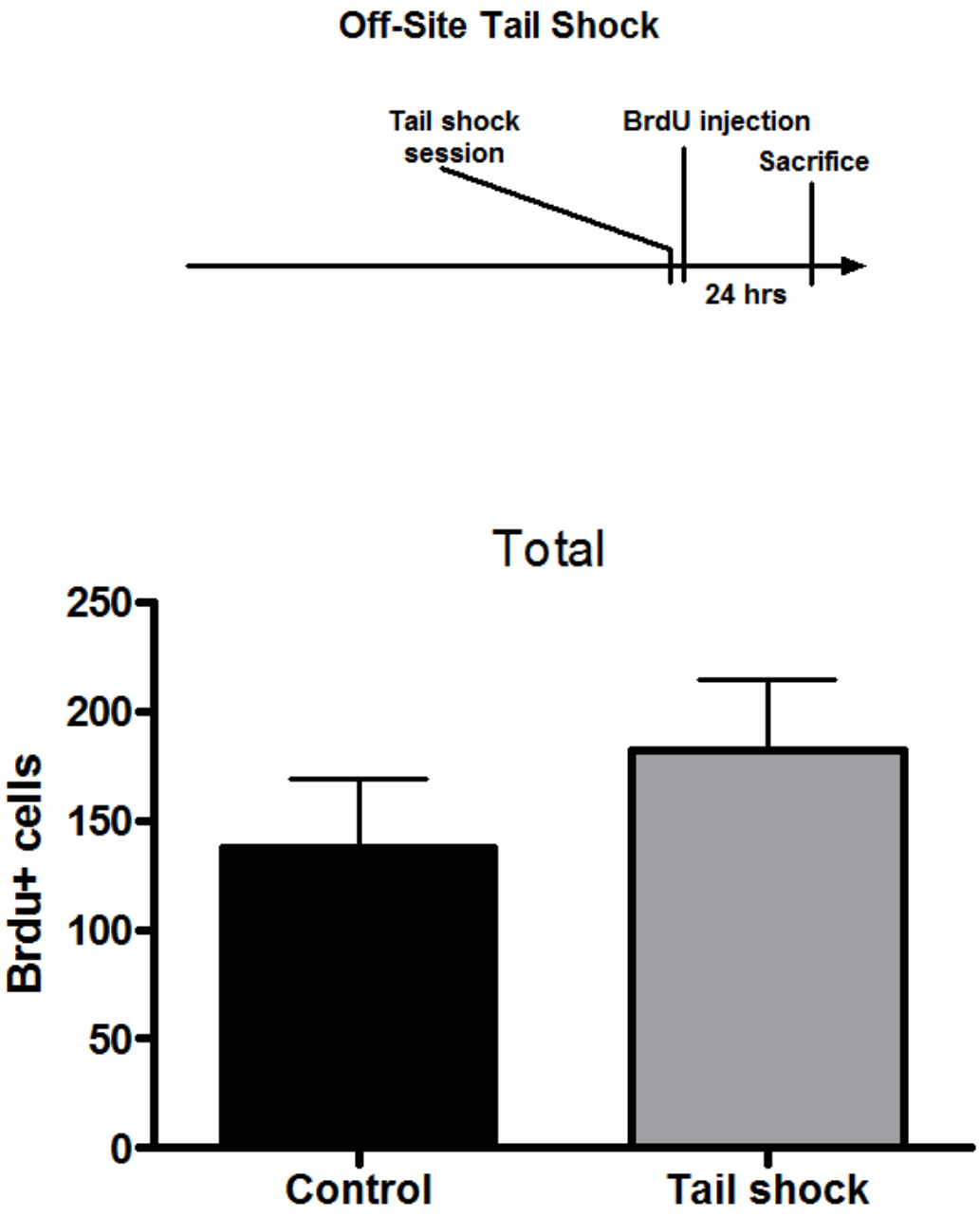

Figure 2-4. BrdU cell counts indicating cell proliferation rate in the dentate gyrus. Data are presented from the entire hippocampus. T-test was not significant. N= 8 per group.

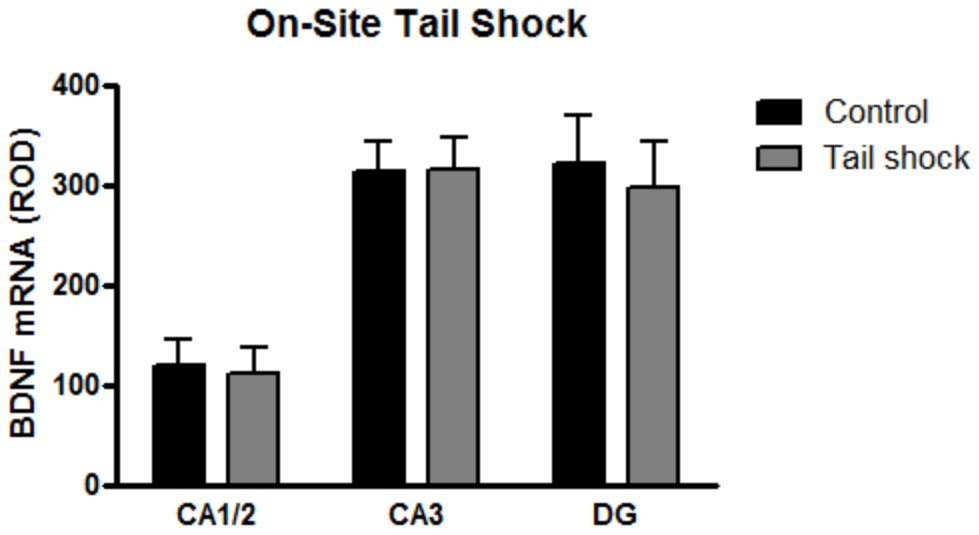

Figure 2-5. *In situ* hybridization of BDNF mRNA. Data are presented as relative optical density (ROD) from the CA1/2, CA3, and dentate gyrus regions. T-tests were not significant for any region. N= 12 per group.

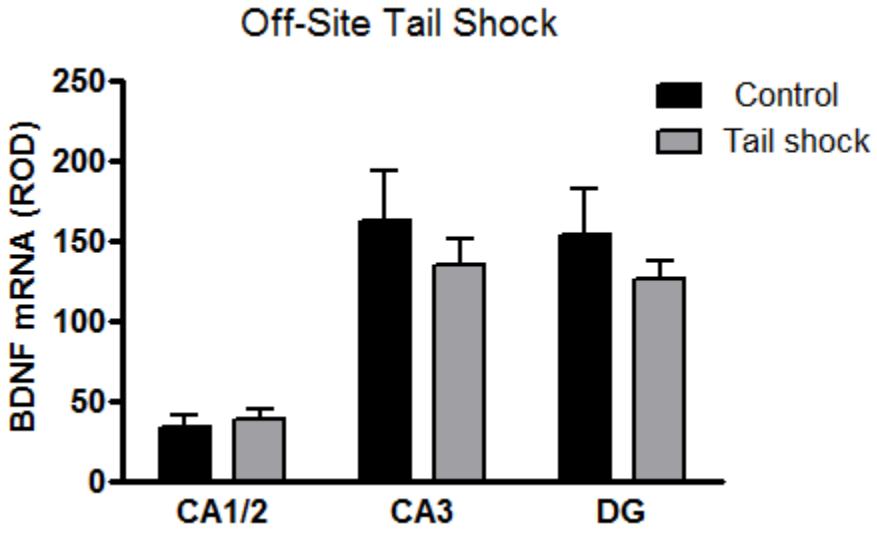

Figure 2-6. *In situ* hybridization of BDNF mRNA. Data are presented as relative optical density (ROD) from the CA1/2, CA3, and dentate gyrus regions. T-tests were not significant for any region. N= 8 per group.

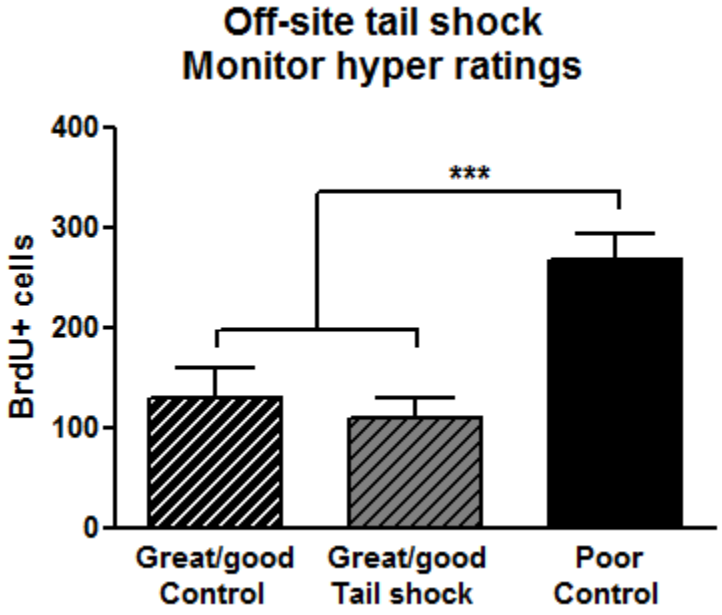

Figure 2-7. BrdU cell counts indicating cell proliferation rate in the dentate gyrus. Data are presented from the entire hippocampus. T-test between hyper rating groups was significant to p=0.0004. N= 4 for the "great/good" control group; n=7 for the "great/good" tail shock group; n=4 for the "poor" control group.

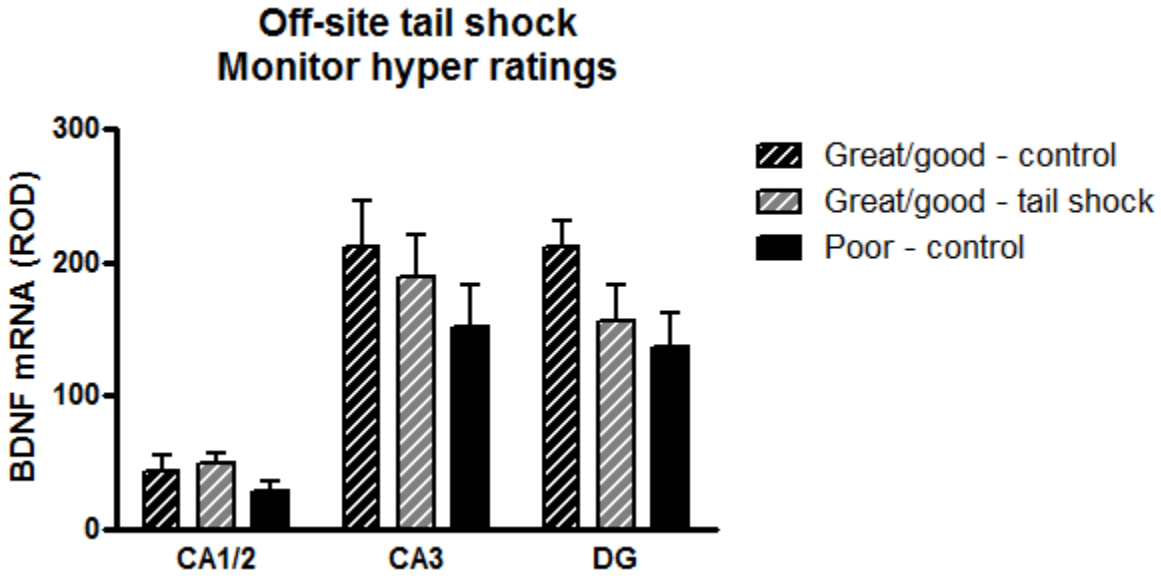

Figure 2-8. *In situ* hybridization of BDNF mRNA. Data are presented as relative optical density (ROD) from the CA1/2, CA3, and dentate gyrus regions. Results were not significant for either "hyper rating" comparison or stress group comparison. N= 4 for the "great/good" control group; n=7 for the "great/good" tail shock group; n=4 for the "poor" control group.

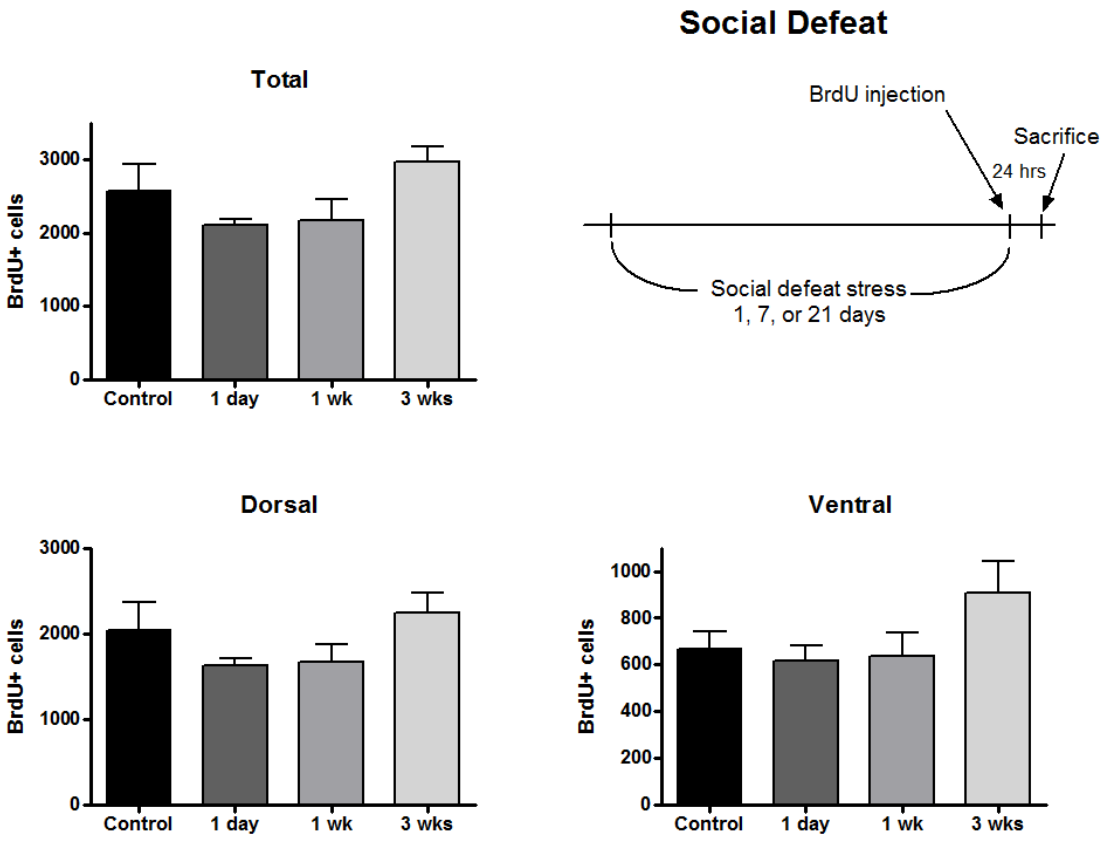

Figure 2-9. BrdU cell counts indicating cell proliferation rate in the dentate gyrus. Data are presented from the entire hippocampus and from the dorsal and ventral regions separated. One-way ANOVA was not significant for any region. N= 6 for each social defeat group; n=8 for control group.

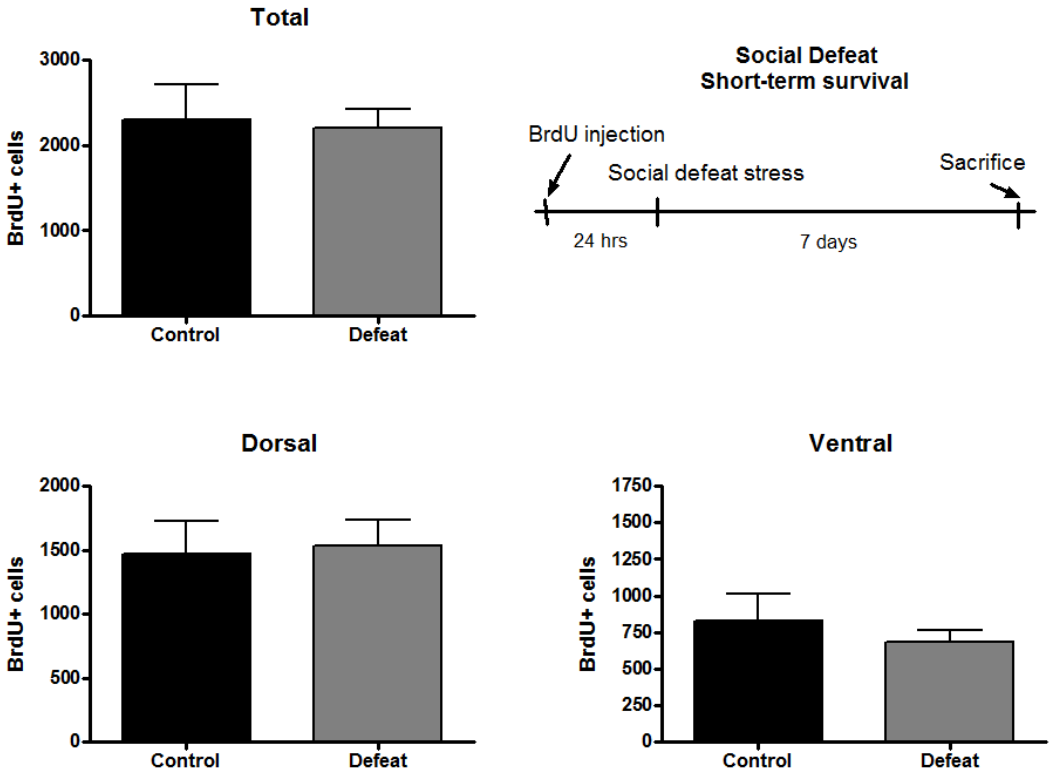

Figure 2-10. BrdU cell counts indicating short term (8 days) cell survival in the dentate gyrus. Data are presented from the entire hippocampus and from the dorsal and ventral regions separated. One-way ANOVA was not significant for any region. N= 12 per group.

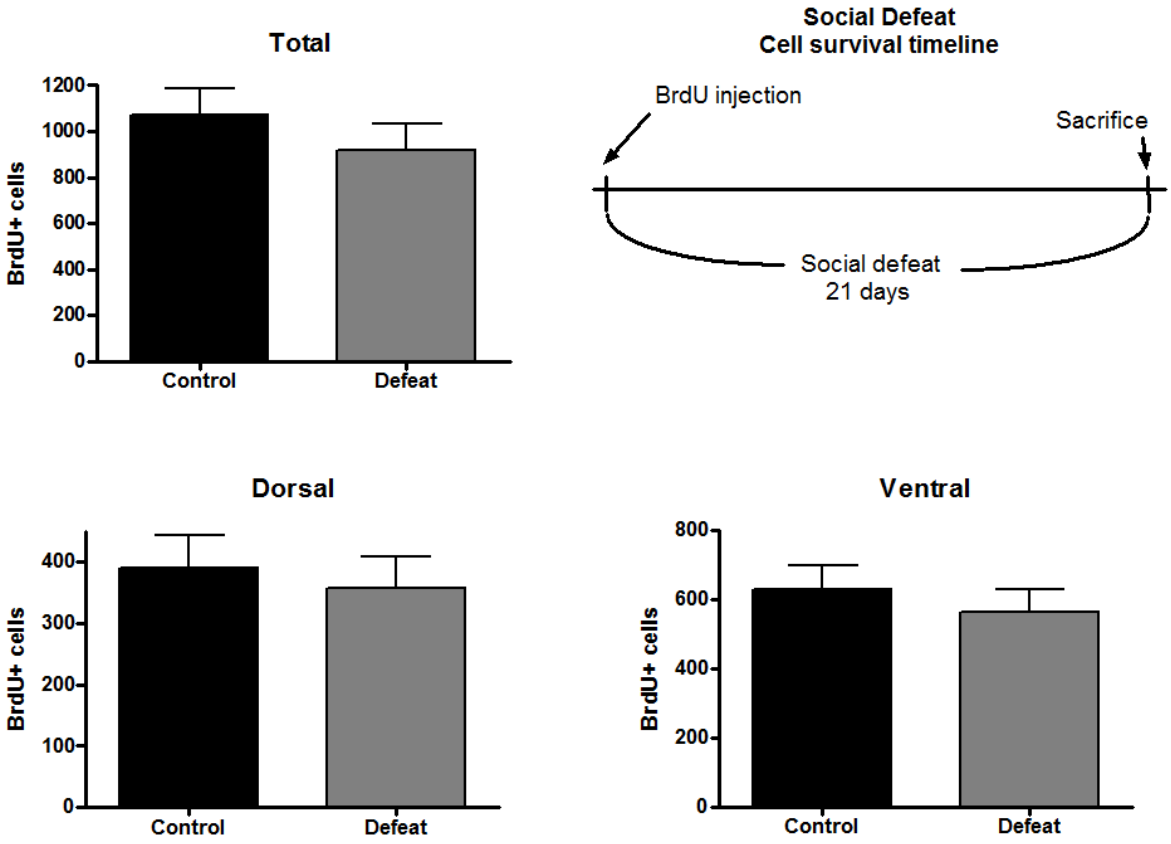

Figure 2-11. BrdU cell counts indicating 21-day cell survival rate in the dentate gyrus. Data are presented from the entire hippocampus and from the dorsal and ventral regions separated. T-tests were not significant for any region. N= 12 per group.

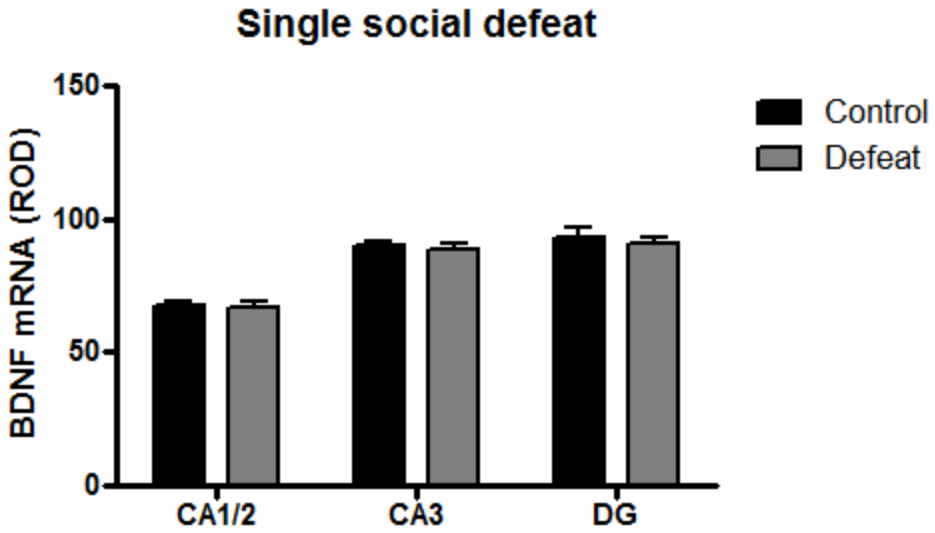

Figure 2-12. *In situ* hybridization of BDNF mRNA. Data are presented as relative optical density (ROD) from the CA1/2, CA3, and dentate gyrus regions. T-test was not significant for any region. N= 12 for each group.

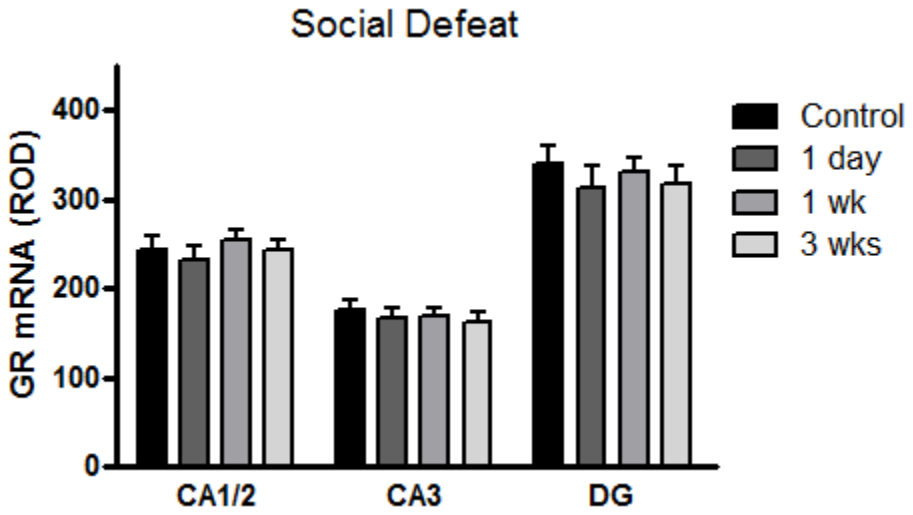

Figure 2-13. *In situ* hybridization of GR mRNA. Data are presented as relative optical density (ROD) from the CA1/2, CA3, and dentate gyrus regions. 1-way ANOVA was not significant for any region. N= 6 for each social defeat group; n=8 for control group.

3: Effects of psychoactive drugs

Monoaminergic-acting antidepressants, as a class, have been hypothesized to upregulate several processes involved in hippocampal neurogenesis, including NPC proliferation, short- and long-term survival of neuroblasts and immature neurons, expression of neurotrophic factors, and growth and branching of neuronal processes. Evidence both supporting and opposing this hypothesis has been reviewed in the introductory chapter. The representative antidepressant used in experiments in this chapter, fluoxetine, was selected due to its wide use in the neurogenesis literature. Several reports have found 14 or more days of fluoxetine treatment to increase both cell proliferation and 28-day cell survival (Malberg, Eisch et al. 2000; Kodama, Fujioka et al. 2004; Marcussen, Flagstad et al. 2008), but failure to confirm these results also exists (Cowen, Takase et al. 2008; David, Samuels et al. 2009).

CRF_1 receptor antagonists, also mentioned in the introductory chapter, are currently being explored clinically as alternatives to conventional monoaminergic antidepressants (reviewed in (Holsboer and Ising 2008)). The CRF_1 antagonist used in experiments here, R121919 (also known as NBI 30775), has been shown to attenuate both behavioral and endocrine responses to stress in rats (Gutman, Owens et al. 2003; Rivier, Grigoriadis et al. 2003). No report of R121919 in neurogenesis has been published, but the CRF_1 antagonist SSR125543 has been reported by two groups to block stress-induced deficits in cell proliferation, although it did not affect cell proliferation under non-stressed conditions (Alonso, Griebel et al. 2004; Surget, Saxe et al. 2008).

Two experiments involving an antidepressant or mood stabilizer augmented with an atypical antipsychotic are included in this chapter. The rationale for performing these experiments arose from clinical evidence of the efficacy of using antipsychotic augmentation in the treatment of refractory bipolar and unipolar depression (Ghaemi and Goodwin 1999; Rothschild 2003). Moreover, both atypical antipsychotics and lithium are effective in augmenting antidepressant non-responders (Simon and Nemeroff 2005; Rapaport, Gharabawi et al. 2006; Keitner, Garlow et al. 2009) and similarly, atypical antipsychotics augment the therapeutic effects of lithium (Bowden 2005). The mechanism(s) underlying these augmenting effects of atypical antipsychotics remain unknown. If neurogenesis plays a role in the therapeutic effects of antidepressants and mood stabilizers, the effects of atypical antipsychotics in augmenting these drugs may also be mediated by neurogenesis.

One study, an inspiration for the experiments in this chapter, reported that olanzapine increased cell proliferation rate in the dentate gyrus with an effect size similar to fluoxetine. However, combination olanzapine-fluoxetine treatment did not increase the rate of neurogenesis above that observed with fluoxetine alone (Kodama, Fujioka et al. 2004). Other atypical antipsychotics reported to increase hippocampal neurogenesis include clozapine, quetiapine, and risperidone (Wakade, Mahadik et al. 2002; Halim, Weickert et al. 2004; Luo, Xu et al. 2005). Furthermore, following chronic restraint stress, quetiapine and venlafaxine exhibit additive effects on neurogenesis (Xu, Chen et al. 2006). However, other studies have detected no effects of atypical

antipsychotics on hippocampal neurogenesis (Wakade, Mahadik et al. 2002; Green, Patil et al. 2006).

The combinations of lithium plus paliperidone (9-hydroxyrisperidone) and escitalopram plus ziprasidone were chosen because of their common clinical use. Chronic lithium treatment has been shown to increase both cell proliferation and survival (Chen, Rajkowska et al. 2000; Son, Yu et al. 2003; Silva, Mesquita et al. 2008). Chronic escitalopram treatment has been shown to increase cell proliferation (Jayatissa, Bisgaard et al. 2006).

Materials and methods

Animals

Adult male Sprague-Dawley rats, approximately eight weeks old (Charles River, Wilmington MA) were obtained one week prior to the starts of the experimental protocols. Animals were maintained in standard laboratory conditions: pair-housed in polycarbonate cages (30 x 20 x 20 cm) with corn cob bedding, on a 12:12 light cycle, with *ad libitum* access to food and water. All procedures used were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University and are in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

Drug administration

Fluoxetine (11 mg/kg as fluoxetine HCl) or R121919 (30 mg/kg) were administered as oral gavage in water once daily for three weeks, with precise dose

matched to each animal's body weight. Control animals were administered the corresponding volume of water. These doses were chosen as both behaviorally active and mimicking therapeutic serum concentrations.

Paliperidone (0.75 mg/kg/day) or vehicle (0.3% tartaric acid) were delivered by subcutaneous osmotic minipumps (Alzet model 2ML4, Durect Corporation, Cupertino CA). After recovering from implantation surgery, animals were administered chow containing 1.2 g/kg lithium carbonate for one week, followed by chow containing 2.4 g/kg lithium carbonate, or control chow (Bio-Serv, Frenchtown NJ). This regimen continued for three weeks total for the cell proliferation experiment and four weeks total for the cell survival experiment. Animals eating lithium chow were provided with 0.9% NaCl in water in order to minimize hyperuria. These doses were chosen to mimic therapeutic serum concentrations.

Escitalopram (2 or 6 mg/kg/day) or vehicle (ethanol / PEG 400) were delivered by subcutaneous osmotic minipumps (Alzet model 2ML4, Durect). After recovering from implantation surgery, ziprasidone (5 mg/kg) was administered as oral gavage in water once daily for three weeks, with precise dose matched to each animal's body weight. Control animals were administered the corresponding volume of water. These doses were chosen to mimic therapeutic serum concentrations (escitalopram) or therapeutic levels of D2 receptor occupancy (ziprasidone).

Serum drug analysis was performed by the lab of Dr. James Ritchie (Emory University) for fluoxetine, lithium, and escitalpram, and by the lab of Drs. Jennifer

Donovan and Lindsay DeVane (Medical University of South Carolina) for paliperidone. Serum concentrations of R121919 and ziprasidone were not measured, due to their short half-lives and unknown identity of active metabolites.

BrdU administration and sacrifice

BrdU (200 mg/kg in 0.9% saline) was administered via a single *ip* injection according to the timelines given for each experiment. For assessment of cell proliferation rate, BrdU injection was given on day 21 or 22 of drug administration and animals were sacrificed 24 (in the fluoxetine and R121919 experiment) or two (in the other experiments) hours later; for assessment of newborn cell survival in the lithium and paliperidone experiment, BrdU injection was given on day 14 or 15 of drug administration and animals were sacrificed 14 days later. At the time of sacrifice, animals were deeply anesthesized with pentobarbital and transcardially perfused with cold 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde. At the time of cardiac puncture, blood was collected for serum drug analysis. The brains were removed, post-fixed overnight, and equilibrated in a 30% sucrose solution. Sections of 25 µm through the entire hippocampus (bregma –2.4 to –6.2) were cut on a cryostat and mounted on slides for ICC and *in situ* hybridization procedures.

Immunocytochemistry

Immunocytochemical staining was performed on every twelfth section throughout the hippocampus. For BrdU staining, antigen retrieval was performed by incubation in 50% formamide/SSC at 65° C followed by 2M HCl at 37° C, then neutralization in 0.1 M boric acid (pH 8.5). After quenching in 2% hydrogen peroxide,

they were blocked in 3% normal horse serum (Vector, Burlingame, CA) and incubated overnight in 1:100 mouse monoclonal anti-BrdU antibody (Becton-Dickinson, Franklin Lakes, NJ). The next day, they were treated with 1:200 horse anti-mouse rat-adsorbed secondary antibody, then treated with a Vectastain ABC kit and visualized with DAB substrate (all Vector).

Stained sections were examined at 40x magnification under a light microscope. Labeled cells were counted if they were within the subgranular zone of the dentate gyrus or within one cell width from its edge. A total number of positively labeled cells was acquired for each animal and multiplied by 12 to approximate the number of labeled cells throughout the entire dentate gyrus. Additionally, cell counts were divided into dorsal and ventral parts of the dentate gyrus. This procedure follows established guidelines for unbiased rare event stereology (Mouton 2002). All cell counting was performed by the same individual under treatment-blind conditions.

A Student's t-test (for each group of the fluoxetine / R121919 experiment) or two-way ANOVA (for the lithium / paliperidone and escitalopram / ziprasidone experiments) was performed on each of the resulting measures, with total dentate gyrus and dorsal and ventral regions considered separately. The criterion for significance was $p < .05$.

In situ hybridization

mRNA was quantified using *in situ* hybridization with an ^{35}S-labeled riboprobe. As in the immunocytological protocol above, every twelfth section was treated and

analyzed. BDNF riboprobe was constructed from cDNA inserts including the full coding regions plus some flanking sequence, ligated into a pR1112-8 plasmid. Radiolabeled antisense cRNA was synthesized by incorporating [^{35}S]-CTP into the probe. The transcription reaction was performed utilizing the Ambion MAXIscript kit with T3 RNA polymerase, according to the instructions provided. Following transcription and removal of the cDNA template with DNase, the cRNA probe was recovered through gel filtration using a G-50 Sephadex Quick Spin column.

The slides underwent 10 minutes of acetylation (0.5% acetic anhydride in 0.1 mol/L triethanolamine, pH 8.0), two rinses in 2X SSC, and dehydration through a graded ethanol series. The sections were then air-dried for at least one hour prior to hybridization. The brain sections were hybridized overnight at 60°C with 1 - 2 x 10^6 cpm of [^{35}S]-labeled cRNA probe diluted into hybridization buffer (50% formamide, 10% dextran sulfate, 0.3 M NaCl, 1X Denhardt's solution, 10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mg/ml yeast tRNA, 10 mM dithiothreitol [DTT]) in humidified Nunc trays.

The next day, slides were allowed to cool to room temperature before being washed four times in 4X saline-sodium citrate buffer (SSC). The sections were then treated with 250 µg/ml RNase A for 30 minutes at 37°C. Subsequently, the slides underwent a series of SSC washes (supplemented with 1 mM DTT) with salt concentrations decreasing from 2X to 0.5X, followed by a 60 minute high stringency wash with 0.1X SSC + 1mM DTT at 60°C, then dehydration through a graded ethanol

series. The slides were air-dried and then apposed to Kodak Biomax MR film until signal reached the desired intensity.

Images on film were digitized with CCD-72 image analysis system equipped with a camera. Semiquantitative analysis was performed using AIS software. Messenger RNA expression levels were calculated by subtracting the neutral background density from the specific signal. Sections were matched for rostrocaudal level and analyzed for values representing the regions CA1/2, CA3, and dentate gyrus, which were then averaged between sections to produce a single value for each region for each animal.

A Student's t-test (for each group of the fluoxetine / R121919 experiment) or two-way ANOVA (for the lithium / paliperidone experiment) was performed on each of the resulting measures, with total dentate gyrus and dorsal and ventral regions considered separately. The criterion for significance was $p < .05$.

Results

Fluoxetine and R121919

In a pilot study, chronic daily dosing of the chosen fluoxetine dosage gave reliable serum concentrations in a narrow range of 600-700 ng/ml fluoxetine plus its active metabolite norfluoxetine at 18 hours post dose. Because the timing of daily dosing for the experiment meant that that animals were past drug nadir at the time of sacrifice, serum concentrations were not assessed for experimental animals.

No effect of either fluoxetine or R121919 on cell proliferation was seen in either the total, dorsal, or ventral dentate gyrus (Figure 3-1; n=12 for each drug group; n=10 for control group).

No effect of either drug on BDNF mRNA expression was seen in any hippocampal area (Figure 3-2; n=12 for each drug group; n=10 for control group).

Lithium and paliperidone

Animals with serum lithium concentrations under 0.40 mM were excluded; serum concentrations of lithium in remaining animals averaged 0.62 mM in the proliferation experiment and 0.65 mM in the survival experiment. Animals with serum paliperidone concentrations under 20 ng/ml were excluded; serum concentrations of paliperidone in remaining animals averaged 69 ng/ml in the proliferation experiment and 66 ng/ml in the survival experiment.

A significant effect of lithium was seen on cell proliferation in all areas ($p<0.0001$ for total; $p=0.0052$ for dorsal; $p=0.0005$ for ventral); no effect of paliperidone was seen (Figure 3-3; n=12 for lithium group; n=8 for each other group). 14-day cell survival showed a significant interaction effect of lithium and paliperidone in the total and ventral measurements ($p=0.0295$ and $p=0.0156$ respectively). There was a trend for increased cell number in the lithium and paliperidone alone groups which decreased to a level similar to control in the lithium/paliperidone combination group (Figure 3-4; n=12 for control and lithium groups; n=8 for paliperidone and pal+li groups).

BDNF mRNA expression was negatively affected by lithium in the CA1/2 and CA3 ($p=0.0100$ and $p=0.0003$, respectively), positively affected by paliperidone in the CA1/2, CA3, and dentate gyrus ($p=0.0002$, $p<0.0001$, $p=0.0060$, respectively), and showed a drug interaction effect in the CA1/2 ($p<0.0001$).

Escitalopram and ziprasidone

Serum concentrations of escitalopram averaged 4.8 ng/ml and 7.9 ng/ml for the low and high dose groups respectively.

No significant effect of either escitalopram or ziprasidone on cell proliferation was seen in either the total, dorsal, or ventral dentate gyrus (Figure 3-6; n=10 per group).

Discussion

The failure of chronic fluoxetine treatment to increase cell proliferation rate was quite surprising. Because the literature on its actions upon neurogenesis indicated that this was a reliable effect, fluoxetine was originally included as a positive control in this experiment. However, after lack of success in producing the expected result, communications with researchers from other laboratories revealed that many had experienced similar difficulties in reproducing what superficially seemed to be an uncomplicated drug response. Most of these negative results have not been published, with the only exceptions involving SSRIs being two studies using fluoxetine and a third using citalopram (Jaako-Movits, Zharkovsky et al. 2006; Cowen, Takase et al. 2008; David, Samuels et al. 2009).

The fact that neither of the SSRIs tested here, fluoxetine or escitalopram, produced changes in cell proliferation despite the use of doses at the high end of those generally producing behavioral effects, suggests that it is not a simple problem with drug administration or a fluke experiment that is responsible for this deviation from the prevailing view of antidepressant effects on neurogenesis. Rather, it is likely that some finer point related to the stress levels or previous experiences of the animals involved determines their responsiveness to antidepressant effects. As detailed in the discussion in the second chapter, these factors might include housing conditions, ambient noise level, personnel activity, conditions related to shipping or breeding colony, or any number of other details which are impossible to entirely control, even at the hands of researchers skilled in minimizing laboratory stress conditions. Early life stress, which would have been under the domain of the breeding facility, has been shown to greatly reduced responsivity of adult neurogenesis to both stress and antidepressant treatment (Mirescu, Peters et al. 2004; Navailles, Hof et al. 2008). Some studies testing the same drugs under both stressed and non-stressed conditions report that an antidepressant regimen which does not alter neurogenesis under non-stressed conditions will fully or partially block the decrease caused by stress (Jaako-Movits, Zharkovsky et al. 2006). This raises the possibility that successful experiments require a certain level of stress and that expertise in minimizing laboratory stress conditions might actually be a hindrance in replicating these experiments. Whatever the key factor might be between laboratories which are able to produce neurogenic effects with antidepressants and

those which are not, it is apparent that alteration of neurogenesis is not a reliable effect of behaviorally active doses of antidepressants.

The failure of the CRF_1 antagonist R121919 to produce an effect, while subject to the same explanations as the antidepressants above, is not so out of line with results predicted by the literature. Of the two reports of the effects of CRF_1 antagonists on cell proliferation under non-stressed conditions, both agreed with this experiment in finding no change (Alonso, Griebel et al. 2004; Surget, Saxe et al. 2008). The additional result showing behavioral antidepressant effects of a CRF_1 antagonist can be achieved even when hippocampal neurogenesis is ablated by x-irradiation or methylazoxymethanol leads to the conclusion that any neurogenic actions of these drugs are not necessary for their antidepressant effects (Surget, Saxe et al. 2008; Bessa, Ferreira et al. 2009). Other recent evidence also supports the hypothesis that neurogenesis may not be itself necessary for antidepressant action and may simply occur in association with other features of neuroplasticity which are closely tied to antidepressant effects (Bessa, Ferreira et al. 2009).

In the lithium and paliperidone experiments, the primary finding was a robust effect of chronic lithium treatment on NPC proliferation rate, while paliperidone had no appreciable effect either alone or as an augmentation to lithium treatment. Interestingly, when the newborn cells were followed to two weeks post-generation with continuing drug exposure, the lithium effect was lost. This suggests that, although lithium increases proliferation rate, there is a corresponding increase in rate of cell

death of this newborn cell population to result in no net gain in new cell number at two weeks of age. One report that chronic lithium treatment increases apoptosis as well as cell proliferation in the dentate gyrus supports this interpretation (Silva, Mesquita et al. 2008). For adult-generated granule cells, the first few weeks of life have been established as crucial in establishing connections to reach functional maturity and enable survival (Dayer, Ford et al. 2003). It may therefore be beneficial to have a larger pool of immature neurons from which the most successfully integrated may be selected, although the resulting number of mature neurons is unchanged.

It should be noted that animals on a lithium diet stabilized at a body weight averaging 10-15% less than animals on a standard chow diet, although they maintained similar standards of general health. This was presumably due to the tendency of animals presented with *ad libitum* chow to eat slightly less (approximately 6%) of the lithium-containing chow, which was was nutritionally equivalent to the control chow (data from pilot animals, not shown). This is common and expected for the dosages given (Dr. Husseini Manji, National Institute of Mental Health, personal communication). There is thus the possibility that the effect of lithium on proliferation rate seen here is not truly a drug effect but is related to the decreased food intake and/or corresponding decreased growth of body mass. There is some evidence for increased neurogenesis under calorie-restricted conditions, but only when total caloric intake is reduced much more than the 6% seen here (Kwon, Jeong et al. 2008).

The failure of paliperidone and ziprasidone to increase either proliferation or survival of new hippocampal neurons was surprising, considering the clinical efficacy of atypical antipsychotics in the treatment of bipolar disorder. This suggests that treatment of bipolar disorder and/or atypical antipsychotic mechanisms involves crucial factors other than neurogenesis, and that these factors along with known monoamine receptors are affected by antipsychotic drugs. The paliperidone-induced increase of BDNF mRNA in the CA1/2 and CA3 of the hippocampus seen here may be one of those factors. As discussed in the introductory chapter, BDNF mRNA expression levels often but do not always correlate with the rate of neurogenesis in the hippocampus, as BDNF/TrkB signaling is theorized to play a positive role in general neuroplasticity (Schmidt and Duman 2007). Here, the disconnect between cell proliferation rate and BDNF expression is glaring, as BDNF expression is increased only in the case of paliperidone treatment alone and normalizes with the addition of lithium.

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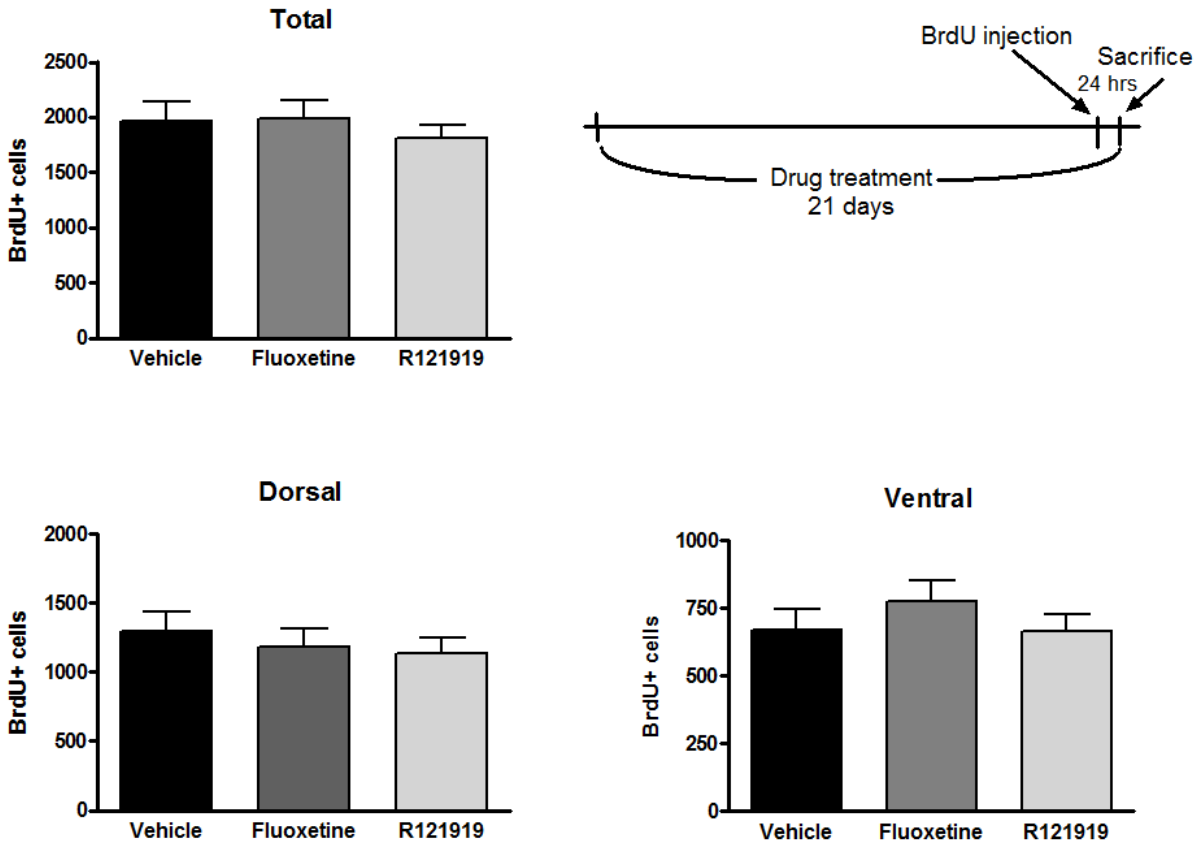

Figure 3-1. Chronic treatment with fluoxetine or R121919. BrdU cell counts indicating cell proliferation rate in the dentate gyrus. Data are presented from the entire hippocampus and from the dorsal and ventral regions separated. Results were not significant for either drug in any region. N=12 for each drug group; n=10 for control group.

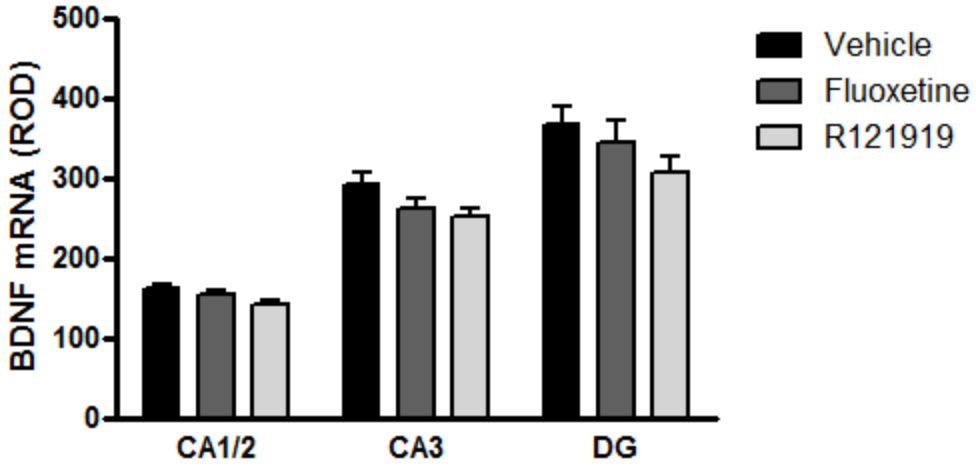

Figure 3-2. Chronic treatment with fluoxetine or R121919. *In situ* hybridization of BDNF mRNA. Data are presented as relative optical density (ROD) from the CA1/2, CA3, and dentate gyrus regions. Results were not significant for either drug in any area. N= 12 for each drug group; n=10 for control group.

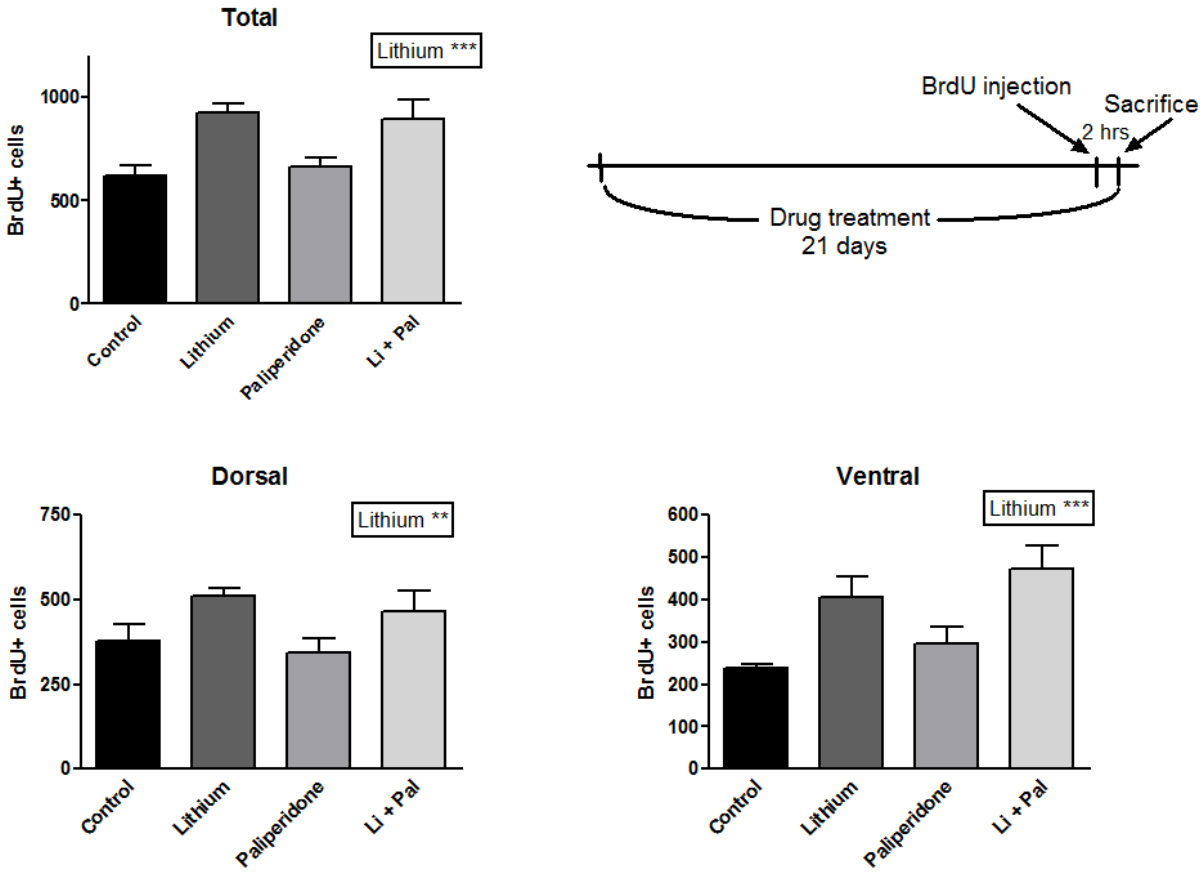

Figure 3-3. Chronic treatment with lithium and/or paliperidone. BrdU cell counts indicating cell proliferation rate in the dentate gyrus. Data are presented from the entire hippocampus and from the dorsal and ventral regions separated. Two-way ANOVA revealed significant effect of lithium in all areas ($p<0.0001$ for total; $p=0.0052$ for dorsal; $p=0.0005$ for ventral). N=12 for lithium group; n=8 for each other group.

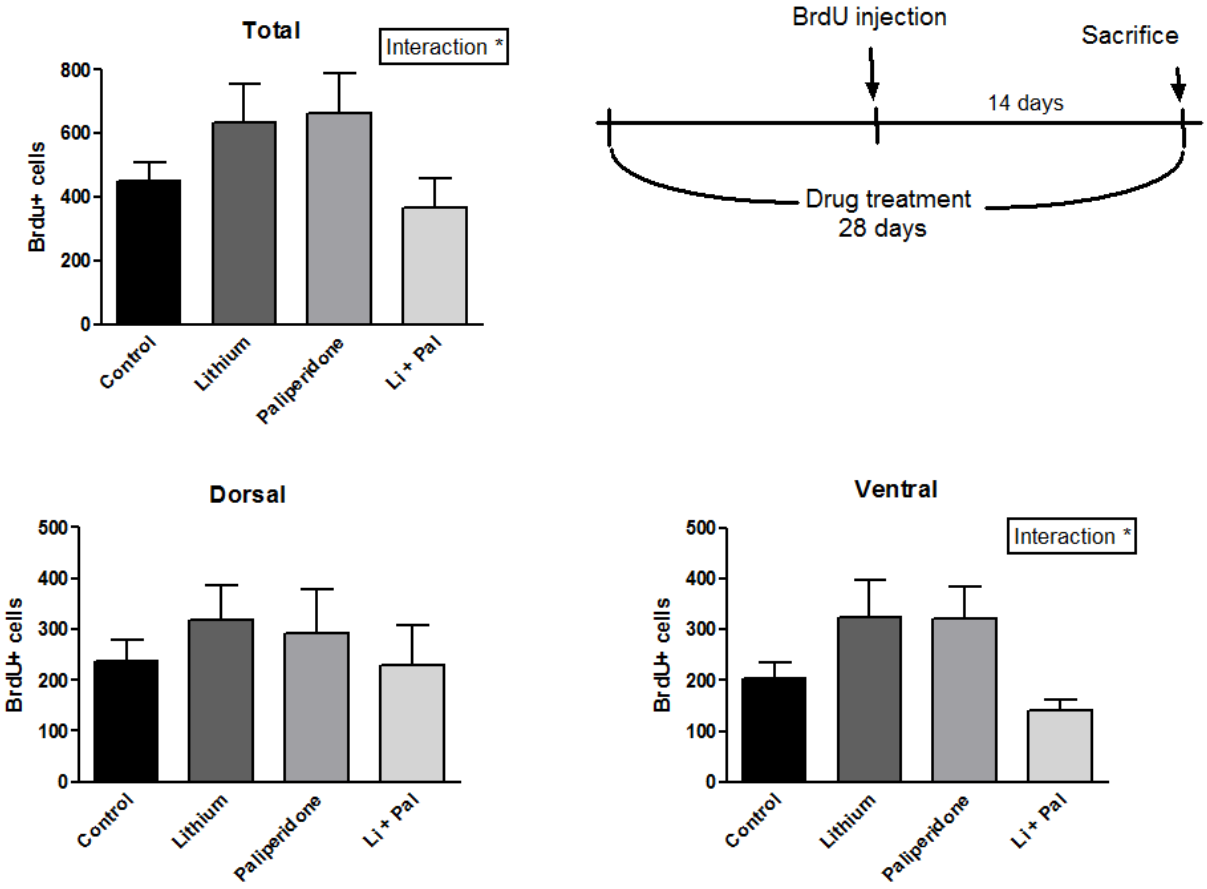

Figure 3-4. Chronic treatment with lithium and/or paliperidone. BrdU cell counts indicating 14-day cell survival rate in the dentate gyrus. Data are presented from the entire hippocampus and from the dorsal and ventral regions separated. Two-way ANOVA revealed significant interaction effect for total and ventral areas (p=0.0295 and p=0.0156 respectively). n=12 for control and lithium groups; n=8 for paliperidone and pal+li groups.

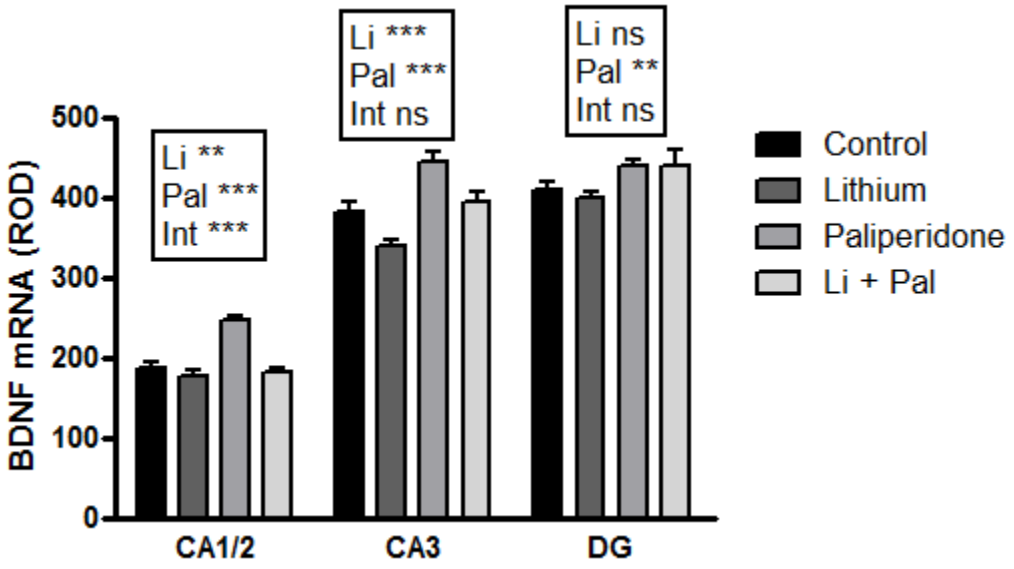

Figure 3-5. Chronic treatment with lithium and/or paliperidone. *In situ* hybridization of BDNF mRNA. Data are presented as relative optical density (ROD) from the CA1/2, CA3, and dentate gyrus regions. Two-way ANOVA revealed significant effects of lithium, paliperidone, and interaction in CA1/2 ($p=0.0010$, $p=0.0002$, $p<0.0001$ respectively); significant effects of paliperidone and interaction in CA3 ($p<0.0001$, $p=0.0003$); and significant effect of paliperidone in the dentate gyrus ($p=0.0060$). N= 12 for lithium group; n=8 for each other group.

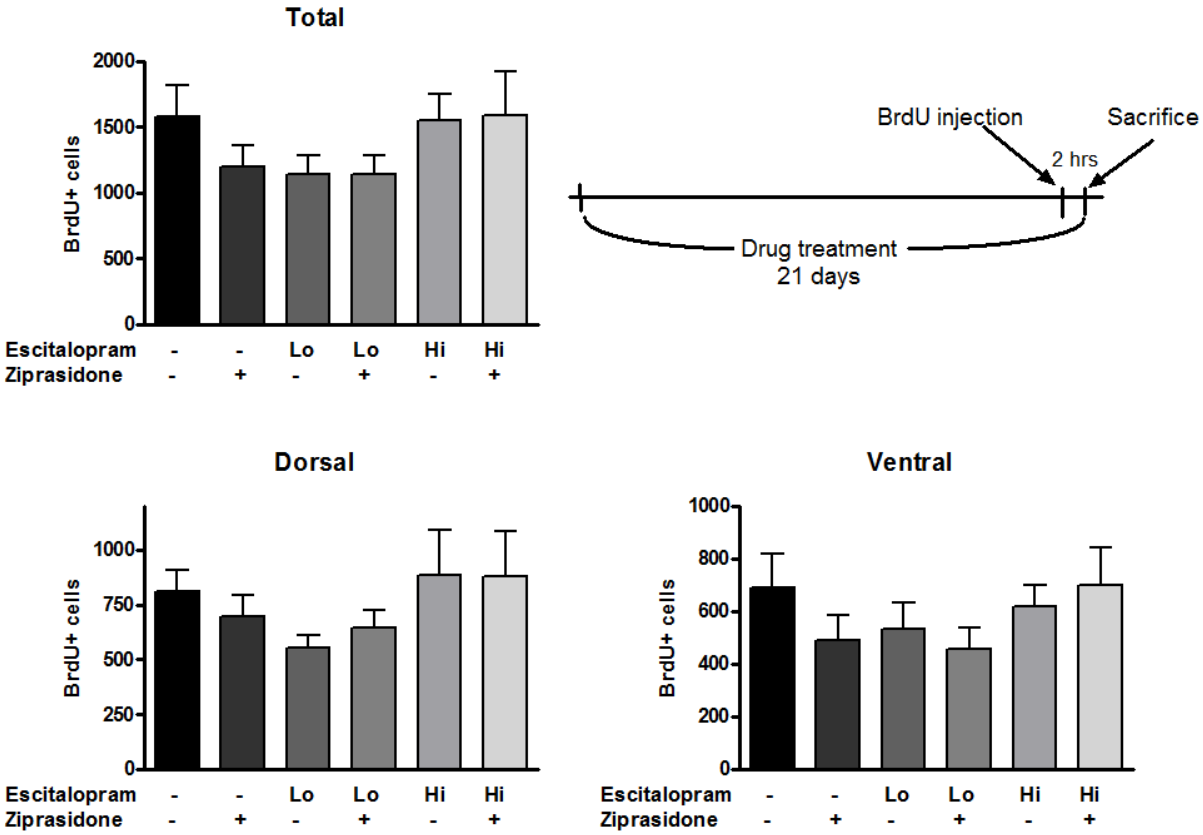

Figure 3-6. Chronic treatment with escitalopram and/or ziprasidone. BrdU cell counts indicating cell proliferation rate in the dentate gyrus. Data are presented from the entire hippocampus and from the dorsal and ventral regions separated. Results were not significant for either drug in any region. N=10 per group.

4: Variations in adolescence

Up to this point, all experiments and discussion have involved neurogenesis in adulthood, primarily in young adulthood but including small forays into older age. This chapter will explore the ways in which the processes and regulation of neurogenesis during the adolescent period may differ from their adult counterparts.

The processes of hippocampal neurogenesis in the adolescent animal differ from those in the adult mainly in magnitude. From birth through the first year, the number of granule cells increases with the volume of the dentate gyrus, maintaining a relatively constant cell density (Bayer, Yackel et al. 1982; Bayer 1985). The NPC proliferation rate approximately triples after birth and slightly increases over the first week of life, reaching its highest level between postnatal days 5 and 8, then falls off after day 10 and continues to slowly decline in a linear fashion into adulthood and through maturity (Schlessinger, Cowan et al. 1975). In normal development, hippocampal BDNF expression is quite low immediately after birth, rises sharply until peaking at postnatal day 14, then remaining steady into adulthood (Das, Chao et al. 2001; Silhol, Bonnichon et al. 2005).

Adolescence, in humans and other primates as well as in rodents, is a period of rapid growth and change, with extensive remodeling still taking place in the stress-responsive regions of PFC, amygdala, and hippocampus (reviewed in (Spear 2000)). Density of serotonin transporter in the frontal cortex increases steadily from the time of weaning throughout adulthood (Moll, Mehnert et al. 2000). It is widely regarded as a

period of enhanced stress sensitivity: adolescent rats have a prolonged corticosterone response to acute stress and a higher peak corticosterone with faster return to baseline after chronic stress when compared with adults (Romeo and McEwen 2006). Some reports find that stress during this period has a greater effect on adult behavior than does the more commonly used early life stress (Tsoory, Cohen et al. 2007; Sterlemann, Rammes et al. 2009).

Considering the increased sensitivity to stress and the accelerated pace of neurogenesis during this stage of development, it seems likely that adolescent animals might show increased sensitivity of neurogenic processes to the influences of stress and/or antidepressant drugs treatment compared with adults. However, studies of neurogenic effects of the types of stress challenges commonly studied in adults are rare, although the enhanced effects of alcohol drinking and dependence in adolescent rats have been well explored, including its enhanced deleterious effects on neurogenic processes during that period (Crews, Mdzinarishvili et al. 2006; Morris, Eaves et al. 2009). One report found that chronic variable stress over the second month of life resulted in reduced volume of the pyramidal layers of the CA1 and CA3 and of the granule cell layer of the dentate gyrus (Isgor, Kabbaj et al. 2004). Studies of antidepressant effects on neurogenesis in adolescents are likewise rare: one study in 30-day-old (at study onset) rats found no effect of chronic fluoxetine treatment on cell proliferation, but also failed to find an effect in young adult or aged cohorts (Cowen, Takase et al. 2008) and another study in similarly aged rats found chronic fluoxetine to

block normal developmental increases in dendritic spine density in the CA1 but not in the dentate gyrus (Norrholm and Ouimet 2000).

The experiments in this chapter, involving chronic social defeat stress and chronic fluoxetine and R121919 administration, were designed as exact replications of experiments earlier performed in adult animals, so that direct comparisons allowing the isolation of age effects would be possible. The period of chronic stress or drug exposure used in these experiments (approximately postnatal day 24 through 43) encompasses not only what is generally considered the adolescent period but also the so-called juvenile period (generally considered postnatal day 21 through 30). The early experimental start was required in order that a full 3 week chronic treatment could be performed while ensuring that the endpoint would be well within the adolescent period and not encroaching upon the onset of adulthood (generally considered postnatal day 60).

Although adolescent rats of the age used in these experiments generally do not engage in territorial competition, they still provoke an aggressive response from trained residents within a social defeat paradigm. Social defeat stress exposures similar to the one used in these experiments have been validated in adolescent cohorts as producing behavioral consequences in line with those produced by social defeat stress in adults, i.e., decreased contact in a social avoidance test, decreased activity in the forced swim test, and increased tendency to develop learned helplessness when confronted with an

escapable shock (Vidal, Bie et al. 2007; Leussis and Andersen 2008; Watt, Burke et al. 2009).

Materials and methods

Animals

Adolescent male Sprague-Dawley rats (Charles River, Wilmington MA) were obtained at 21 days old, two days prior to the starts of the experimental protocols. This short lag time between animal delivery and experiment onset was necessary so that chronic (3 week) experiments could be carried out within the adolescent period without subjecting the animals to early weaning. Animals were 42 -43 days old at the time of sacrifice. Resident animals for the social defeat procedures were male Long-Evans retired breeders (Charles River), pair-housed with sexually mature females.

In all cases, animals were maintained in standard laboratory conditions: pair-housed (except as noted for social defeat cohorts) in polycarbonate cages (40 x 28 x 20 cm for social defeat residents; 30 x 20 x 20 cm for all others) with corn cob bedding, on a 12:12 light cycle, with *ad libitum* access to food and water. All procedures used were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University and are in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

Social defeat stress

Animals subjected to social defeat were housed individually, in order to maximize the impact of the defeat stress by removing the soothing effects of non-

hostile conspecifics and to eliminate the additional variable of social dominance or subordinance in the home cage pair. Control animals were housed in pairs.

Several weeks in advance of the social defeat experiment, resident animals were trained for aggressive territorial behavior by challenging them multiple times with smaller intruder animals in the social defeat procedure detailed below. Only those animals which met criteria for sufficiently aggressive and dominant behaviors were used as residents in the social defeat experiments. These criteria included minimum latency to first attack and minimum latency to pin.

Between 2:00 and 4:00 pm, resident animals were transported to another room, where the female cagemates were removed, and were left undisturbed for one hour. Experimental animals in the defeat group were then transported from the colony and each one placed into the homecage of a resident, separated by a wire mesh barrier which allowed visual and olfactory contact only for the first 2 minutes of the encounter. The barrier was then removed and the animals allowed to interact for 20 minutes. If the intruder was injured during the encounter, the barrier was replaced for the remainder of the session. Sessions took place on Mondays, Wednesdays, and Fridays, with resident-intruder pairs systematically shuffled for each session to equalize the defeat experience between experimental animals. Behavior was monitored during these sessions, and any intruder which did not display sufficient defeated behavior was omitted from the experimental analysis.

Drug administration

Fluoxetine (11 mg/kg as fluoxetine HCl) or R121919 (30 mg/kg) were administered as oral gavage in water once daily for three weeks, with precise dose matched to each animal's body weight. Control animals were administered the corresponding volume of water. These doses were chosen as both behaviorally active and mimicking therapeutic serum concentrations.

BrdU administration and sacrifice

BrdU (200 mg/kg in 0.9% saline) was administered via a single *ip* injection according to the timelines given for each experiment. For assessment of cell proliferation rate, BrdU injection was given immediately following the final stress session and animals were sacrificed 24 hours later. At the time of sacrifice, animals were deeply anesthesized with pentobarbital and transcardially perfused with cold 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde. The brains were removed, post-fixed overnight, and equilibrated in a 30% sucrose solution. Sections of 25 µm through the entire hippocampus (bregma –2.4 to –6.2) were cut on a cryostat and mounted on slides for ICC and *in situ* hybridization procedures.

Immunocytochemistry

Immunocytochemical staining was performed on every twelfth section throughout the hippocampus. For BrdU staining, antigen retrieval was performed by incubation in 50% formamide/SSC at 65° C followed by 2M HCl at 37° C, then neutralization in 0.1 M boric acid (pH 8.5). For Ki-67 staining, antigen retrieval was performed by incubation in 10 mM citrate buffer at 90°C. After quenching in 2%

hydrogen peroxide, they were blocked in 3% normal horse serum (Vector, Burlingame, CA) and incubated overnight in 1:100 mouse monoclonal anti-BrdU antibody (Becton-Dickinson, Franklin Lakes, NJ) or 1:100 mouse monoclonal anti-Ki-67 antibody (Vector). The next day, they were treated with 1:200 horse anti-mouse rat-adsorbed secondary antibody, then treated with a Vectastain ABC kit and visualized with DAB substrate (all Vector).

Stained sections were examined at 40x magnification under a light microscope. Labeled cells were counted if they were within the subgranular zone of the dentate gyrus or within one cell width from its edge. A total number of positively labeled cells was acquired for each animal and multiplied by 12 to approximate the number of labeled cells throughout the entire dentate gyrus. Additionally, cell counts were divided into dorsal and ventral parts of the dentate gyrus. This procedure follows established guidelines for unbiased rare event stereology (Mouton 2002). All cell counting was performed by the same individual under treatment-blind conditions.

A Student's t-test (for the social defeat experiment) or two-way ANOVA (combining data from the two iterations of the drug experiment) was performed on each of the resulting measures, with total dentate gyrus and dorsal and ventral regions considered separately. The criterion for significance was $p < .05$.

Additionally, when BrdU and Ki-67 cell counts were available for the same animal cohorts, these individual points, ungrouped by treatment, were subjected to

correlations analysis (Pearson's r). As both numbers represent the same population of proliferating cells, these numbers should correlate strongly.

In situ hybridization

mRNA was quantified using *in situ* hybridization with an ^{35}S-labeled riboprobe. As in the immunocytological protocol above, every twelfth section was treated and analyzed. BDNF and GR riboprobes were constructed from cDNA inserts including the full coding regions plus some flanking sequence, ligated into pR1112-8 (BDNF) or pGemA (GR) plasmids. Radiolabeled antisense cRNAs were synthesized by incorporating [^{35}S]-CTP into the probes. The transcription reactions were performed utilizing the Ambion MAXIscript kit with T3 RNA polymerase for BDNF or T7 RNA polymerase for GR, according to the instructions provided. Following transcription and removal of the cDNA template with DNase, the cRNA probes were recovered through gel filtration using a G-50 Sephadex Quick Spin column.

The slides underwent 10 minutes of acetylation (0.5% acetic anhydride in 0.1 mol/L triethanolamine, pH 8.0), two rinses in 2X SSC, and dehydration through a graded ethanol series. The sections were then air-dried for at least one hour prior to hybridization. The brain sections were hybridized overnight at 60°C with 1 - 2 x 10^6 cpm of [^{35}S]-labeled cRNA probe diluted into hybridization buffer (50% formamide, 10% dextran sulfate, 0.3 M NaCl, 1X Denhardt's solution, 10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mg/ml yeast tRNA, 10 mM dithiothreitol [DTT]) in humidified Nunc trays.

The next day, slides were allowed to cool to room temperature before being washed four times in 4X saline-sodium citrate buffer (SSC). The sections were then treated with 250 µg/ml RNase A for 30 minutes at 37°C. Subsequently, the slides underwent a series of SSC washes (supplemented with 1 mM DTT) with salt concentrations decreasing from 2X to 0.5X, followed by a 60 minute high stringency wash with 0.1X SSC + 1mM DTT at 60°C, then dehydration through a graded ethanol series. The slides were air-dried and then apposed to Kodak Biomax MR film until signal reached the desired intensity.

Images on film were digitized with CCD-72 image analysis system equipped with a camera. Semiquantitative analysis was performed using AIS software. Messenger RNA expression levels were calculated by subtracting the neutral background density from the specific signal. Sections were matched for rostrocaudal level and analyzed for values representing the regions CA1/2, CA3, and dentate gyrus, which were then averaged between sections to produce a single value for each region for each animal.

A Student's t-test (for the social defeat experiment) or two-way ANOVA (combining data from the two iterations of the drug experiment) was performed on each of the resulting measures, with total dentate gyrus and dorsal and ventral regions considered separately. The criterion for significance was $p < .05$.

Results

Social defeat stress

After 21 days (9 sessions) of social defeat stress, average body weight of the defeat group was 8% lower than that of the control group (p=0.0082).

No significant effect of social defeat stress on cell proliferation rate was seen, in either in the entire dentate gyrus or in the dorsal or ventral parts (Figure 4-1; n=9 for control group; n=12 for defeat group).

Expression of BDNF and GR mRNA were also unchanged (Figures 4-2 and 4-3).

Fluoxetine and R121919

Doses of fluoxetine and R121919 were identical to those used in the adult experiment reported in chapter 3, which were there validated as being behaviorally active and, for fluoxetine, mimicking clinical therapeutic serum values. The dose-serum relationship for fluoxetine has been experimentally shown to be equivalent in adults and adolescents (Dr. Gretchen Neigh, Emory University, personal communication).

Chronic administration of fluoxetine and R121919 was performed in two identical experiments. Cell proliferation rate was assessed using both BrdU and Ki-67. For each of these, two-way ANOVA analysis (drug x experiment) was performed for each drug for each of the total, dorsal, and ventral hippocampal regions. No effect on proliferation rate was found for either drug in any area, as measured by BrdU (Figure 4-4; n=8-10 per group) or by Ki-67 (Figure 4-5; n=8-10 per group).

Correlation analysis of Ki-67 vs BrdU cell counts revealed a strong linear correlation ($r=0.5461$; $p<0.0001$) with a slope of 0.2669 ± 0.05423.

Discussion

The defeat paradigm was validated as stressful by behavioral responses of the experimental animals to resident aggression (as in adults) and additionally by the significantly reduced weight gain of animals in the defeat group over the course of the experiment, an effect which was not seen in adults. Despite this, no greater effect of social defeat stress on NPC proliferation rate or on BDNF or GR mRNA expression was seen in adolescents than in adult animals. The unchanged expression of GR mRNA after a chronic stress exposure, a result identical to that seen in adults, is consistent with recent reports in adolescent animals (Romeo, Ali et al. 2008).

As in adult animals, no effect was found in adolescents of chronic treatment with either fluoxetine or R121919 on cell proliferation. This was disappointing but less surprising than the lack of response of adolescent cell proliferation to chronic stress, as the only published report of antidepressant effect on adolescent neurogenesis is a negative one (Cowen, Takase et al. 2008).

The cell proliferation rate in adolescent animals was, as expected, substantially higher than in adult animals (approximately 5000 vs. approximately 3000 BrdU-labeled cells after a 24-hour incorporation period) *cf.* (Schlessinger, Cowan et al. 1975).

The 60 animals from both adolescent drug experiments also served as validation of the BrdU incorporation, staining, and counting methods against Ki-67 labeling as an

accurate representation of cell proliferation rate. Sections from each animal, without regard to treatment group, were independently subjected to ICC and stereological cell counting for both markers. These two measurements correlated extremely strongly, in accordance with the idea that both provide estimates of the same population of dividing cells. However, Ki-67+ cell counts were almost four times as high as BrdU+ cell counts. If Ki-67, an endogenous marker believed to be expressed in all cycling cells (Kee, Sivalingam et al. 2002), indicates the true size of the proliferating NPC population, the BrdU labeling protocol used in these studies only reveals a portion of the population it is designed to represent. This incomplete labeling is most likely caused by systemic BrdU being metabolized and/or excreted in a timeframe shorter than the NPC cell cycle (Cameron and McKay 2001). The strong linear correlation between BrdU+ and Ki-67+ cell counts suggests that this proportion is constant, meaning that although the BrdU+ cell counts in these studies are not quantitatively accurate portrayals of the cycling population, they are valid for comparing rates of proliferation between groups.

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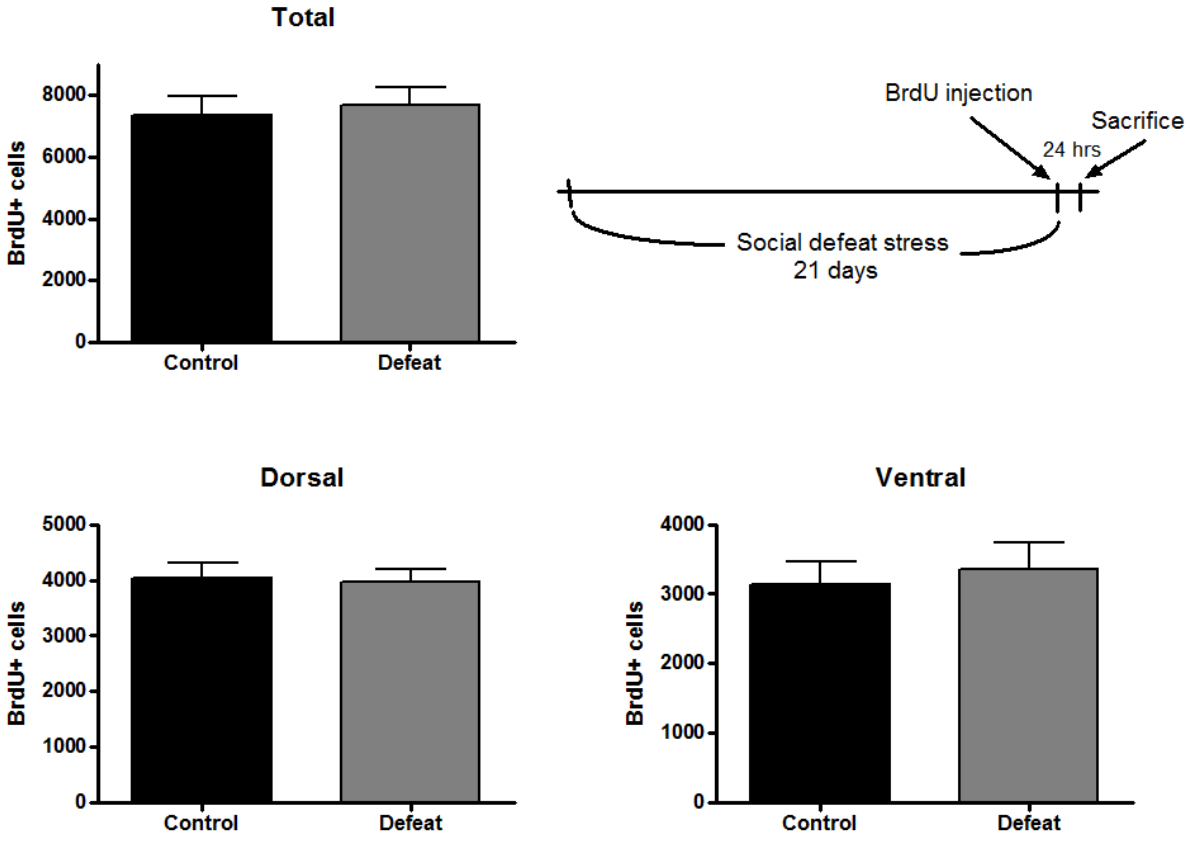

Figure 4-1. BrdU cell counts indicating cell proliferation rate in the dentate gyrus. Data are presented from the entire hippocampus and from the dorsal and ventral regions separated. Groups were not significantly different for any region. n=9 for control group; n=12 for defeat group.

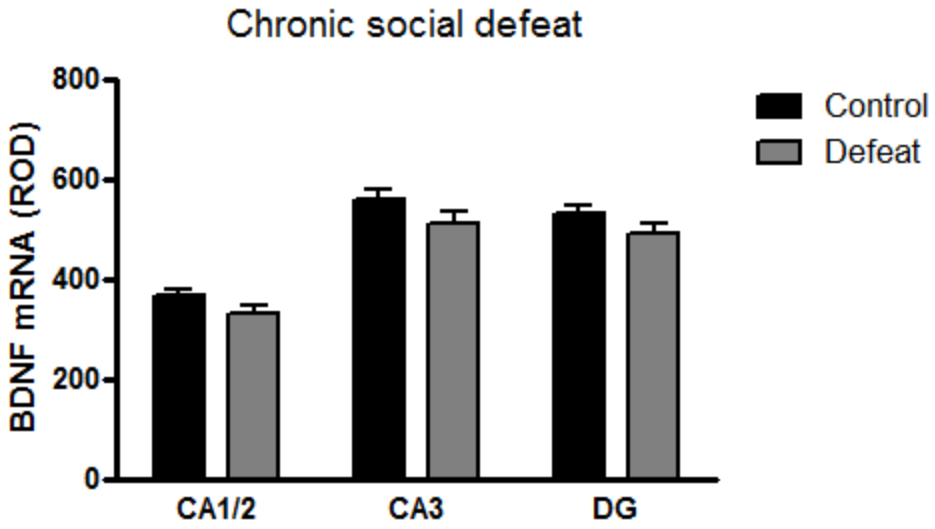

Figure 4-2. Chronic social defeat stress in adolescent animals. *In situ* hybridization of BDNF mRNA. Data are presented as relative optical density (ROD) from the CA1/2, CA3, and dentate gyrus regions. Groups were not significantly different in any area. N= 12 per group.

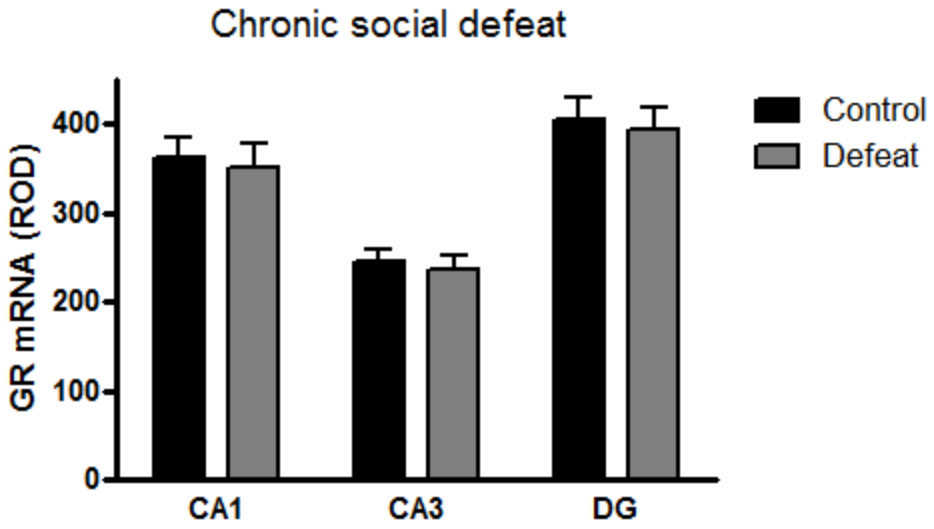

Figure 4-3. Chronic social defeat stress in adolescent animals. *In situ* hybridization of GR mRNA. Data are presented as relative optical density (ROD) from the CA1/2, CA3, and dentate gyrus regions. Groups were not significantly different in any area. N= 12 per group.

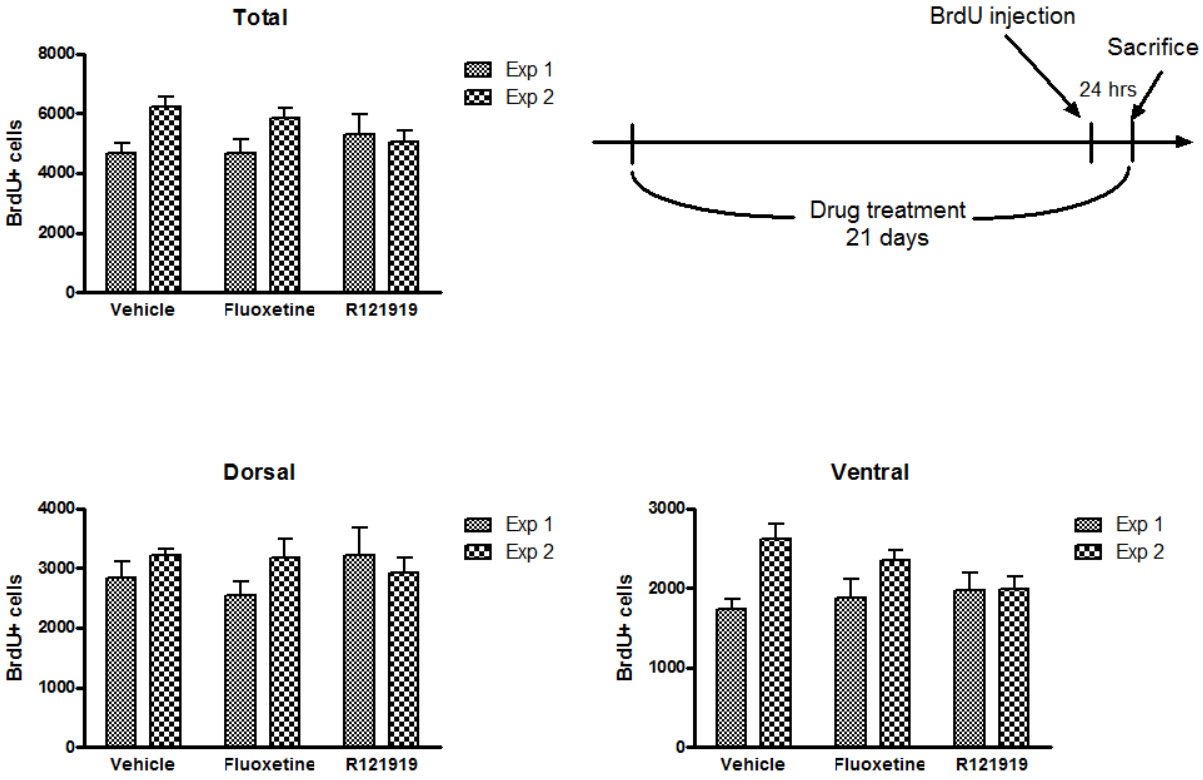

Figure 4-4. Chronic treatment with fluoxetine or R121919 in adolescent animals, for two identically performed experiments. BrdU cell counts indicating cell proliferation rate in the dentate gyrus. Data are presented from the entire hippocampus and from the dorsal and ventral regions separated. Neither drug produced a significant effect in any area. N=8-10 per group.

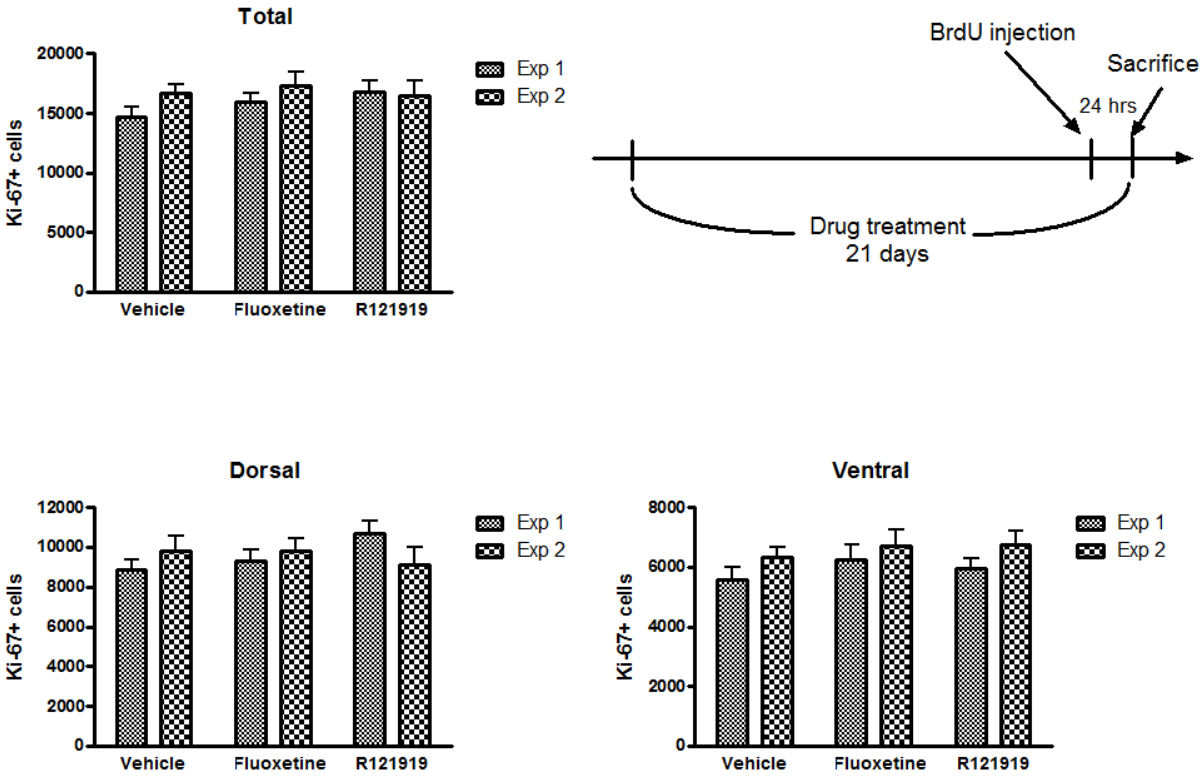

Figure 4-5. Chronic treatment with fluoxetine or R121919 in adolescent animals, for two identically performed experiments. Ki-67 cell counts indicating cell proliferation rate in the dentate gyrus. Data are presented from the entire hippocampus and from the dorsal and ventral regions separated. Neither drug produced a significant effect in any area. N=8-10 per group.

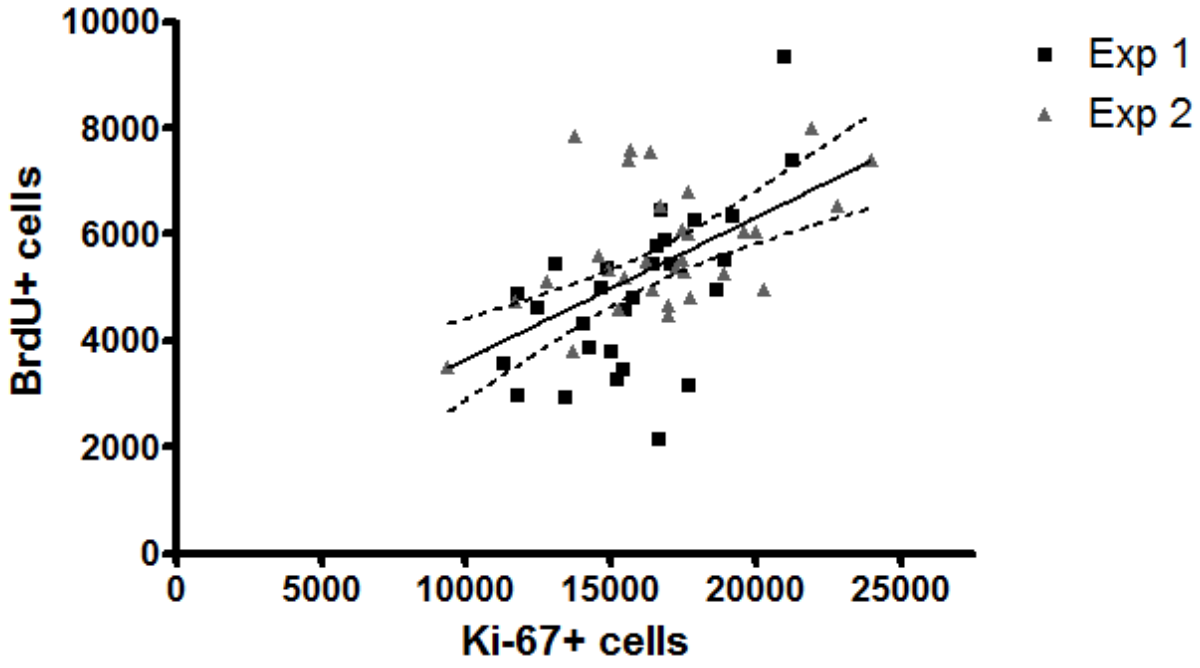

Figure 4-6. Correlation of individual BrdU+ and Ki-67+ cell counts from adolescent fluoxetine/R121919 experiments. 60 combined data points correlate to r=0.5461 ($p<0.0001$).

5: General discussion

The original aim of this project was to expand understanding of hippocampal neurogenesis and its associated processes and regulation by analyzing its responses to manipulation of the CRF system. At that time, the available body of published research indicated that a simple rubric held true: hippocampal neurogenesis (particularly, the proliferation rate of NPCs located in the dentate gyrus) was downregulated by any sufficiently intense and prolonged psychological stress and upregulated by chronic administration of a several classes of antidepressant drugs, including SSRIs. Moreover, there was a strong body of evidence suggesting that deficits in hippocampal neurogenesis might play an integral part on the psychopathology of major depression and that restoration of these deficits might be a necessary component of the therapeutic effects of antidepressants.

The difficulties encountered in effecting any significant variation in NPC proliferation rate have meant a dramatic departure of the ultimate content of this project from what was initially proposed and have even led to questioning of the prevailing hypotheses regarding regulation of adult neurogenesis.

Stress and neurogenesis

As detailed in the second chapter, “Effects of stress”, this project failed to recreate the downregulation of NPC proliferation rate reported by numerous laboratories to result from restraint, shock, or social stress. It is extremely unlikely that the negative results reported here, covering three distinct stress modalities, the specific

protocols of which have been validated by previous use within this and other laboratories including endocrine and behavioral measures, are the result simply of insufficiently stressful experimental procedures. Additionally, the method used here of assessing the population of cycling NPCs using BrdU injection and immunocytological labeling has been demonstrated by correlation with Ki-67 labeling (in chapter 4) to provide a valid proportional representation of the true population, and (in the case of the lithium effect reported in chapter 3) to be capable of indicating an experimentally induced change in that population when one exists. Thus, the conclusion naturally arises that stress *per se* is not sufficient to inhibit cell proliferation, and that what seems in many cases to be an effect of stress must actually be either an effect of some factor often but not always associated with experimental stress procedures or an epiphenomenon related to but not essentially linked to the true stress response or, more, likely, both.

The first case includes consideration of the usual confounds which come into play in behavioral research. These are detailed in the discussion section of chapter 2 and include, briefly, variation in the level of stress which is considered “baseline” in association with housing conditions and animal handling, unknowability of animal experience prior to arrival in the experimental facility, and aspects of fear learning and memory (such as place- or contextual-cued conditioning) involved in the experimental stress procedures. The studies which are most illuminating in this regard are neither the ones which produce the most robust and reliable stress-induced deficits in neurogenesis nor the ones (like the experiments in this project) which produce the most baffling lack

of these results, but the ones which use subtle manipulations of experimental procedure within a laboratory to isolate those factors which distinguish a stressor capable of disrupting neurogenesis from one which is not. One elegant example of this is found in a series of experiments in which the ability of a footshock stressor to decrease cell proliferation is dependent upon animals having a five minute pre-exposure to the shock chamber (Pham, McEwen et al. 2005). Another is the repeated finding that escapable footshock does not produce the same neurogenic deficits found after inescapable footshock (Malberg and Duman 2003; Shors, Mathew et al. 2007).

The second case represents a concern less with the stress-neurogenesis link in favor of a focus on teasing out what components of hippocampal plasticity are directly linked with stress and depressive psychopathology (Vollmayr, Mahlstedt et al. 2007). Dendritic arborization and synaptic remodeling are two areas which have begun to drawn attention away from new neuron production. Both are inhibited by prolonged stress and recovered in association with antidepressants (Vyas, Mitra et al. 2002; Tata, Marciano et al. 2006; Bessa, Ferreira et al. 2009). These processes can be observed in pyramidal cells of the CA1 and CA3 as well as dentate granule cells, and to a lesser degree in prefrontal cortex, relieving the conundrum posed by the neurogenesis hypothesis: why the hippocampus should be the brain region most important in depression when changes seen in the depressed brain are widespread across so many systems (Goldapple, Segal et al. 2004). The factors which mediate alterations in these structural changes are a complicated target, as it is less likely that a single molecule plays an essential role than that an extensive cast of players create an environment

accommodating to neuroplasticity. One of the most popular candidates in this field, BDNF, did not show changes in the experiments performed here, but many other hippocampal signaling factors have been shown to be regulated by stress, such as NCAM (Nacher, Gomez-Climent et al. 2004; Sandi, Woodson et al. 2005), and synapsin (Magarinos, Verdugo et al. 1997; Wu, Han et al. 2007).

Antidepressants and neurogenesis

The failure of antidepressants to increase cell proliferation from a non-stressed baseline (detailed in chapter 3) is a less daunting question, easier to reconcile with the literature than is the failure to find stress effects. A number of studies employing antidepressants of various classes have found that they do not alter neurogenesis under non-stressed conditions, but only serve to rescue a deficit caused by experimental stress (Jaako-Movits, Zharkovsky et al. 2006; David, Samuels et al. 2009). Such results are compatible with the clinical ability of antidepressants to alter mood in depressed patients while not causing changes in normal subjects. Additionally, CRF_1 antagonists have never been shown to affect cell proliferation from a non-stressed baseline (Alonso, Griebel et al. 2004; Surget, Saxe et al. 2008). The unavailability of an effective neurogenesis-reducing stressor in this project meant that no experiments regarding the power of these drugs to block that reduction could be performed. A logical inference might be that a small amount of environmental stress, even in an animal cohort considered to be unstressed, might be present in many of the published reports of antidepressant enhancement of neurogenesis, and that the ability of a laboratory to maintain very low stress levels might be detrimental to the replication of these results.

A case has been set forward that hippocampal neurogenesis is required for the therapeutic actions of antidepressants; the major caveat to this argument is that there is no definitive rodent model of depression. When NPC proliferation is blocked, either via chemical agents or x-irradiation, antidepressants remain active in the forced swim test but not in the novelty-suppressed feeding test; both are considered measurements of depressive behavior and are widely used as screening tests for antidepressant efficacy (Santarelli, Saxe et al. 2003; Bessa, Ferreira et al. 2009; David, Samuels et al. 2009). Interestingly, experiments involving the Flinders sensitive line, a genetic model of depression which under normal conditions exhibits reduced neurogenesis, show antidepressants increasing NPC proliferation, bringing the animals closer to normal function (Chen, Madsen et al. 2009; Petersen, Wortwein et al. 2009). This suggests that antidepressants may not act as facilitators of neurogenesis under all conditions, but only serve to normalize it in situations where it has become perturbed, much as their clinical effects on mood are only seen in depressed patients and not in normal subjects.

As is the case with effects of stress on neurogenesis, it is likely that antidepressants facilitate a general environment of neuroplasticity, which may include the upregulation of neurogenesis, rather than acting directly and primarily on neurogenesis.

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