PROZAC AND PUBERTY: THE EFFECTS OF FLUOXETINE TREATMENT ON NEUROGENESIS IN THE PUBESCENT HIPPOCAMPUS

by

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ABSTRACT OF THE DISSERTATION

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Chronic treatment with fluoxetine (Prozac) increases cell proliferation and neurogenesis in the dentate gyrus of the adult male rat (Encinas et al., 2006; Malberg et al., 2000). Therefore neurogenesis was proposed to be a mechanism through which antidepressants alleviate some symptoms of depression (Jacobs et al., 2000; Duman, 2004a; Sapolsky, 2004). Here we tested whether chronic fluoxetine treatment increases cell proliferation and neurogenesis in pubescent and adult female rats. Rats were injected with fluoxetine (5mg/kg) or saline for 14 days. One day later subjects were injected with 5-bromo-2'-deoxyuridine (BrdU; 200mg/ kg), a marker of dividing cells. Rats in experiment 1 were sacrificed 2hrs after BrdU injection to measure cell proliferation. Subjects in experiment 2 were sacrificed at 24 hrs to measure cell proliferation after a full cell cycle. In experiment 3 subjects were sacrificed 28 days after BrdU injection to measure neurogenesis. At all time points fluoxetine increased proliferation and neurogenesis in

adult male rats. However, the drug did not alter proliferation or neurogenesis in pubescent males. Proliferation and neurogenesis were elevated during puberty for both sexes, irrespective of treatment. There were no effects of fluoxetine treatment on proliferation or neurogenesis in females at either age, or across the estrous cycle. Immunofluorescent labeling with BrdU and NeuN a marker of mature neurons verified an effect of fluoxetine treatment only in adult male subjects. Double labeling also indicated that cell fate was not altered at any age by treatment. Blood samples were taken to determine whether fluoxetine altered circulating levels of sex and stress hormones 24 hrs or 29 days after treatment. Treatment decreased corticosterone concentrations in pubescent female rats sacrificed at the later time. There were no other hormonal effects of fluoxetine treatment. These data indicate that pubescent rats respond differently to antidepressants than adults. Hormonal changes in the pubescent female seem especially susceptible to the effects of fluoxetine. In addition, fluoxetine may operate differently in adult females than in adult males. Together, these results indicate that antidepressants probably operate via different neuronal mechanisms in adult males than in other age groups or even the other sex.

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INTRODUCTION

In 2004 the FDA released a public health advisory that treatment with the selective serotonin reuptake inhibitor (SSRI) class of antidepressants increased the incidence of suicidal thought and behavior in adolescents (FDA warning, 2004). In the same warning they announced that fluoxetine, brand name Prozac, was the only antidepressant approved for use in children and adolescents. The initial warning was based on a meta-analysis conducted by the FDA utilizing 24 short-term placebo – controlled studies, but continuing research now includes a total of 372 studies (Kuehn, 2007). Part of the difficulty in determining whether adolescents should be treated with SSRIs is due to the use of human participants in these studies, which introduce confounding variables. While it is impossible to determine whether animals experience suicidal thoughts, it is possible to determine whether these drugs act differently on the pubescent brain. Therefore the following experiments were conducted to determine whether the SSRI class of antidepressant fluoxetine has the same biological impact on pubescent rats as it does in adults. To determine this, we used the physiological indicator of neurogenesis in the dentate gyrus of the hippocampus, as recent research has postulated a role for neurogenesis in the etiology of depression.

Neurogenesis

Before discussing the role of neurogenesis in depression it is necessary to describe the process of neurogenesis in the hippocampus. New neurons are thought to arise through mitotic division from progenitor cells residing in the subgranular zone (SGZ) in the dentate gyrus. These daughter cells then migrate to the granular cell layer where they

mature and are incorporated. As adult granule cell neurons they send axons known as mossy fibers to pyramidal cells in the CA3 region of the hippocampus which in turn synapse on to pyramidal cells in area CA1 (for review see Christie & Cameron, 2006).

Several techniques have been developed to visualize and characterize these cells. The most common techniques use exogenous markers of DNA synthesis such as Bromodeoxyuridine (BrdU) a thymidine analog, and [^{3}H] Thymidine which are then visualized using peroxidase methods or in the later case autoradiography. Additionally endogenous markers such as Ki67 and proliferating cell nuclear antigen (PCNA) have also been developed to measure cell proliferation. These markers label all stages of the cell cycle except $G_{0,}$ whereas the exogenous marker BrdU only labels cells in the S phase of the cell cycle. In addition some studies have utilized viral vectors to visualize new cells. This technique allows visualization of the entire cell through its development, but this benefit is contrasted by variability in label quality, reduction in the number of labeled cells and the necessity of using a surgical technique to inject the viral vector. A variety of these markers can also be co-labeled with immunofluorescent markers of immature or mature neurons to determine that the labeled cells display a neuronal phenotype (for review see Arbrous et al., 2005; Christie & Cameron, 2006; Taupin 2006).

Divisions are also made for the relationship between injection of the mitotic marker and subsequent sacrifice of the subject. For BrdU a two hour time point is considered proliferation as the marker is available for uptake in to cells undergoing DNA synthesis for 2hours. Therefore a single cohort of cells undergoing the S phase of the cell cycle is labeled without marking cells undergoing apoptosis (Nowakowski et al., 1989). Longer time periods such as 4 hrs- 4 days allow visualization of re-dividing and post

mitotic cells but also account for cells undergoing a full cell cycle. The cell cycle in the mouse has been calculated to take ~13 hr (Hayes & Nowakowski, 2002) and ~24 hrs in the rat (Cameron & McKay 2001). Sacrifice at 1-4 weeks allow visualization of immature and mature neurons which can be co-labeled with the appropriate markers (Dayer et al., 2003).

A series of experiments by Van Praag et al. (2002) described the time course and subsequent incorporation of new cells into the hippocampus. Mice were injected with green florescent protein virus (GFP) and sacrificed 48 hours, 4 weeks, or 4 months after injection. Using GFP to visualize the cells they found that at 48 hours cells were located in the inner granule cell layer, of these cells 50 % were found to display co-labeling for immature neurons or glia. None displayed co-labeling for mature neurons, and 50% were uncharacterized. At 4 weeks cells were localized in the granular cell layer with dendritic processes extending towards the molecular layer and axons projecting to the hilar area. These cells were co-labeled with adult neuronal markers. The new cells co-expressed labeling for synaptophysin a marker of active synapses and had presynaptic vesicles which faced postsynaptic densities. Cells also possessed dendritic spines, which are considered terminals for glutametergic inputs. At 4 months cells had grown in terms of soma size, dendritic arbors and spine density by 60 %, and appeared similar to normal adult granule cells. The authors also provided evidence through in vitro electrophysiological recordings that these cells had all of the characteristics of mature cells. Therefore it was determined that neurons arise, migrate and then if they survive, are incorporated over the next 4 weeks into the granular cell layer. Over the next 4 months they fully mature and for all appearances become normal granule cell neurons.

Neurogenesis and depression

The neurogenesis theory of depression is based on evidence of hippocampal atrophy determined by imaging and post mortem studies in depressive patients (Sheline, 1996; Stockmier et al., 2004 ; Videbech & Ravnkilde, 2004 but see Vythilingham et al., 2004). Meta analysis of imaging studies determined that depressive patients have a 9% decrease in hippocampal volume (Videbech & Ravnkilde, 2004). It was proposed that this volumetric decrease is related to a decrease in neurogenesis and overall hippocampal plasticity and that antidepressant treatment reverses this deficit and restores normal function (Duman, 2004a; Jacobs et al., 2000; Jacobs 2002; Sapolsky, 2004; Vollmayr et al., 2004). It should be noted that a recent report that utilized an endogenous marker of proliferation in human post-mortem tissue determined that cell proliferation was not reduced in major depression but was reduced in schizophrenia (Reif et al., 2006). This study did not differentiate between medicated and un-medicated depressives, a possible confounding factor if antidepressants increase cell proliferation in humans. The majority of the evidence for a role of neurogenesis in depression comes from studies of the relationship between neurogenesis and SSRIs. These include similarities in the time course of incorporation and maturation of newly arisen granule cells to the time lag in efficacy of treatment (Sapolsky, 2004; Van Praag et al., 2002). The necessity of serotonin for neurogenesis (Brezun et al., 1999 but see Huang & Herbert, 2005b), along with the ability of serotonin based treatments to reverse stressed based reductions in neurogenesis (Chen et al., 2006 ;Malberg & Duman, 2003 but see Vollmayr et al., 2003) and increase cell proliferation and neurogenesis (Malberg et al., 2000; Encinas et al., 2006). In

addition there has been recent evidence indicating that neurogenesis is necessary condition for the action of SSRI treatment on novelty suppressed feeding (Santarelli et al., 2003). However this study is somewhat controversial as the behavioral measure used as a model of depression is not widely accepted. A recent study which decreased cell proliferation in the hippocampus by partial serotonergic denervation produced no behavioral effects on the forced swim test a more recognized measure of depression like behavior (Rosenbrock et al., 2005).

A few caveats exist for the neurogenesis theory of depression. First the decrease in hippocampal volume reported for depressive patients is less than the variability (12-14%) which occurs naturally in humans (Lupien et al., 2007). In addition most rodent studies determined that the decrease in neurogenesis as a result of inescapable shock does not result in an overall decrease in hippocampal volume (Chen et al., 2006; Malberg & Duman, 2003). Finally the rate of cell proliferation in rats (~4000 per day in 4 m male rats of which ~3000 were neurons) and mice (~ 9000 cells per day) is much higher than the rate of cell proliferation in primates and accounts for a larger percentage of replacement in the granule cell layer (~200 per day or 0.04% of the GCL) (Cameron & Mckay, 2001; Kornack & Rackic, 1999; Rao & Shetty 2004). Therefore importance of neurogenesis as a mechanism for depression and hippocampal function in humans is questionable at best.

Neurogenesis and stress

While the relationship between neurogenesis and depression is still unclear, the effects of stress on neurogenesis have been robustly demonstrated in adult male rodents

(Cameron & Gould, 1994; 1998). Removal of the adrenal glands increased neurogenesis and subsequent application of exogenous corticosterone decreased neurogenesis in adrenalectomized and sham operated rats (Cameron & Gould, 1994). In addition it was determined that corticosterone (CORT) reduced cell proliferation by acting on NMDA receptors. Previously the same group had demonstrated that both NMDA and CORT manipulations separately affected cell proliferation (Cameron & Gould, 1994; Gould et al., 1992). This group of experiments demonstrated that both manipulations act on the same pathway, and the NMDA action is downstream of the CORT action. Prior to puberty males and females take longer to return to baseline levels of CORT after stressor exposure than adults (Romeo et al., 2004; Romeo et al., 2005). Given that this difference is on average twice the length of time that adults take, it is likely that there is an added effect of stress on neurogenesis prior to puberty and possibly during puberty.

Stressful experience reduces cell proliferation and neurogenesis in adult male rats (Tanapat et al., 2001). Exposure to fox odor but not a neutral odor reduced the number of new cells produced in the dentate gyrus at 2 hour and 1 week survival points. The reduction in proliferation was blocked by adrenalectomy. However exposure to fox odor did not decrease proliferation in females who were freely cycling or ovariectomized (with and without estradiol replacement), suggesting that females may be immune from the effects of stress on proliferation (Falconer and Galea, 2003). Further evidence for this supposition was generated by the report that acute foot shock decreases cell proliferation in adult male but not female rats compared to naïve controls (Shors et al., 2007). Additionally the paper demonstrated that chronic uncontrollable stress decreased cell proliferation in adult male rats compared to controllable stress, but the type of stressor

did not affect female cell proliferation. In a separate group of subjects males subjected to chronic uncontrollable stress displayed learned helplessness behavior when tested in an FR2 shuttle box escape avoidance task, whereas females subjected to the same treatment did eventually learn the task, albeit slower than their yoked controls exposed to controllable stress. Together these studies suggest that stress decreases cell proliferation in adult male but not adult female rats.

In adult subjects SSRIs block stress induced impairments in behavior related to neurogenesis. Treatment of at least 7 days with fluoxetine blocked the deficit in escape avoidance for rats exposed to inescapable shock (Malberg & Duman, 2003). In a separate group of rats the experimenters demonstrated that this period of treatment also blocked the stress related decrease in cell proliferation. However the relationship between learned helplessness and proliferation did not utilize the same subjects as the active avoidance task itself reduced neurogenesis in subjects who did not engage in learned helplessness behavior compared to levels in naïve controls. A study by a different group did use the same subjects for behavioral testing of learned helplessness and neurogenesis (Chen et al., 2006). This study exposed subjects to inescapable stress of tail shock on days 1 and 7, and used a shuttle box active avoidance task to measure learned helplessness on days 2 and 8. A subset of the learned helplessness rats were then treated for one week with fluoxetine or desipramine and all subjects were retested on day 14 after which they were injected with BrdU and sacrificed on day 15. The researchers demonstrated that untreated stressed rats had a longer latency to escape than their unstressed counterparts. Additionally they demonstrated that fluoxetine treatment decreased the escape latency in helpless rats, whereas desipramine only partially reversed the deficit. Finally they

determined that subjects displaying learned helplessness behavior had decreased cell proliferation, and fluoxetine treatment reversed this deficit. However a different study (Vollmayr et al., 2003) found no relationship between the decrease in proliferation and learned helplessness. They determined that 45 minutes of immobilization stress decreased cell proliferation, but did not affect learned helplessness behavior. In addition they exposed rats to a total duration of 20 minutes of foot shock in a 40 minute session. After all rats had been exposed to inescapable shock they were tested in an active avoidance task. The researchers then compared BrdU labeled cells in subjects that displayed helplessness behavior and those that did not. They determined that there were no differences in the amount of cell proliferation for the 2 groups; it should be noted that this study had methodological issues and lacked the proper controls as all subjects were exposed to a great deal of inescapable stress. The most compelling evidence so far for a relationship between neurogenesis, SSRIs and behavior was a study by Santarelli et al. (2003) which determined that irradiation of the sub-granular, but not the sub-ventricular zone blocked the behavioral effects of fluoxetine on a novelty suppressed feeding paradigm. They also demonstrated that chronic, but not acute treatment with a variety of antidepressants shortened the latency to feed in the same behavioral paradigm. However as previously mentioned novelty suppressed feeding is not a normally accepted model of depression, but is generally thought of as an anxiety task.

Neurogenesis in females

The vast majority of human and animal studies relating to depression have been conducted in males, despite the fact that depression is twice as likely to occur in women

as men (Earls, 1987; Kessler et al., 2003). Neurogenesis in female rats is affected by the stage of the estrous cycle and the presence of ovarian hormones (Tanapat et al., 1999; 2005). Female rats in proestrus, a stage when estrogen and progesterone levels are rising to their peak, had higher levels of neurogenesis than females in the other stages of their estrous cycle and males. Estrogen replacement after ovariectomy also increased cell proliferation; however treatment 48 hours later with progesterone reduced cell proliferation to similar levels as ovariectomized females. The estrogen induced increase in cell proliferation is dependent on the presence of serotonin (5HT) (Banasr et al., 2001). Estrogen did not increase cell proliferation in rats that were 5HT depleted by p-chlorophenylalanine (PCPA). Interestingly depletion of 5HT through lesion or limiting its production decreases cell proliferation in females (Brezun and Daszuta, 1999; 2000) but not males (Huang & Herbert, 2005b). However as no single study has directly compared the sexes and different strains were used in the above mentioned studies, it is possible that other factors may have influenced these findings.

Other aspects of hippocampal plasticity such as dendritic spine density are also influenced by the estrous cycle (Woolley, 1998; Woolley & McEwen, 1993; Woolley et al., 1990). Dendritic spine densities are increased during the proestrus stage of the cycle compared to the other stages. Furthermore there is evidence that SSRIs affect spine density in adult ovariectomized female rats (Hajszan et al., 2005). Five days of treatment with fluoxetine increased spine synapse density by 68.8% in area CA1; longer treatment (14 days) increased spine synapse density in both areas CA1 and CA3 by greater than 60%.

Three papers have examined the effects of SSRIs on neurogenesis in female mice. In an examination of the role of brain derived neurotrophic factor (BDNF) in the actions of antidepressant drugs on proliferation and cell survival (Sairanen et al., 2005) the researchers averaged both sexes together, apparently finding no sex differences in the effects of antidepressants on proliferation or cell survival. However, as this study used very small numbers of males and females, per group (4 of each sex) and were not looking specifically at sex differences, it is possible that they did not have enough statistical power to find any significant sex difference. More compelling evidence comes from a recent study in which male and female mice were given 21 days of fluoxetine treatment (Lagace et al, 2007). The study found that chronic treatment with 10mg/kg of fluoxetine increased cell proliferation in the SGZ for both male and female mice. Unlike the rat they did not find any effects of ovariectomy or the estrous cycle on cell proliferation in females (Tanapat et al., 1999; Tanapat et al., 2005). However a third report examining only female mice determined that doses of 5 or 10mg/kg did not increase cell proliferation, but a dose of 25mg/kg did increase cell proliferation (Engesser-Cesar et al., 2007). Therefore it is still unclear as to whether these drugs increase proliferation and neurogenesis in female rats as they do in males (Encinas et al., 2006; Malberg et al., 2000).

SSRIs and hormones

Both chronic and acute treatment with SSRIs increase basal levels of corticosterone (CORT) in male rats (Serra et al, 2001; Weber et al., 2006 but see Stout et al., 2002). However, males treated chronically with fluoxetine and exposed to an acute stressor of foot shock had similar increases in CORT responses to saline treated controls

(Serra et al., 2001). Fluoxetine treatment does not increase corticosterone concentrations in adult female rats (Van de Kar et al., 2002). In general female rats have higher levels of corticosterone than males at least during the proestrus stage of the cycle (Atkinson & Waddell, 1997). In prepubescent male rats chronic treatment with fluoxetine did not alter basal levels of CORT or ACTH (Landry et al., 2005), however these subjects were not compared to adult controls, so we do not yet know if this is an actual difference in corticosterone response to fluoxetine or due to differences in experimental methods. Therefore it is necessary to determine whether chronic fluoxetine treatment increases basal corticosterone levels in pubescent rats as it does in adult male rats.

Diurnal rhythms of corticosterone release are necessary for the effects of fluoxetine treatment on neurogenesis (Huang & Herbert, 2006). Flattening of the diurnal rhythm through adrenalectomy and/or replacement of corticosterone through corticosterone pellets blocked the effects of fluoxetine on cell proliferation. Additionally mimicking the diurnal rhythm through a pellet implant of 15% corticosterone accompanied by a 2mg/kg injection of corticosterone at the beginning of the dark cycle, reinstated the fluoxetine induced increase in cell proliferation.

SSRIs decrease sexual behavior in both the male and female rat (Matuszczyk et al., 1998; Taylor et al., 1996, 2004). However the relationship between this behavioral impairment and a hormonal basis is still relatively unclear. For males the decrease in sexual behavior during chronic treatment with fluoxetine was not related to testosterone, or other measures of male sexual physiology (Taylor et al., 1996), whereas a different study indicated that higher doses of fluoxetine did decrease testosterone levels (Rygula et al., 2006). Sub-chronic administration of fluoxetine impaired female sexual behavior

without affecting the actual estrous cycle in freely cycling rats (Matuszczyk et al., 1998). Whereas other studies have reported that fluoxetine treatment disrupted the estrous cycle and sexually receptivity (Uphouse et al., 2006). A report utilizing microdialysis of the mediobasal hypothalamus determined that untreated female rats have higher levels of 5HT during the proestrus stage of the cycle than at any other stage of the cycle or males during daylight conditions (Maswood et al., 1999). However reverse dialysis with fluoxetine caused a 4 fold increase of 5HT levels in estrus females and males compared to a slight increase in proestrus and diestrus females, suggesting that the female brain is less receptive to alterations due to fluoxetine treatment at certain stages of its cycle. It should be noted however that this study examined direct acute application of fluoxetine to the brain rather than systemic chronic treatment.

Serotonergic innervation of the anterior and medial hypothalami by the dorsal raphe nucleus (DRN) has been indicated as important to the onset of puberty in the female (Monroy et al., 2003). Complete lesions of the DRN resulted in blockade of first ovulation and a decrease in progesterone. Addition of propranolol, a beta adrenergic blocker increased progesterone and lead to ovulation. Estrogen levels were not affected by DRN lesions. Taken together these studies suggest that there is an interaction between female sex hormones and 5HT which may differently affect neurogenesis in the female rat.

Neurogenesis and puberty

Puberty is a developmental period involving changes in the body, such as the emergence of secondary sexual characteristics (Ojeda & Urbanski, 1994). It is also

marked by changes in the nervous system, particularly volumetric changes in limbic brain regions and axonal reorganization (Geidd et al., 1996; 1997; Gogtay et al., 2004; Paus et al., 1999). Behaviorally we have demonstrated that stress effects on learning emerge during puberty, whereas sex differences emerge after puberty (Hodes & Shors, 2005). Given the degree of change during puberty, it is hypothesized that psychotropic drugs would act differently during puberty than adulthood.

While no studies have directly examined the relationship between puberty, antidepressants and neurogenesis, a few have examined levels of neurogenesis produced during puberty in untreated conditions. Initially Cameron and McKay (2001) examined the effects of low doses of BrdU on rats in the early stages of puberty (5 weeks). They found dose dependent differences in the number of labeled cells both 24 hours and 4 weeks after injection. This effect was not found in the adult subjects used for the rest of their experiments. More interestingly a large dose of 300 mg/kg labeled almost double the number of cells in pubescents than in adults when examined at the 24 hour time point indicating that pubescent rats have more cell proliferation than adults. A second study using a different dose (50mg/kg three injections separated by 2 hours intervals) determined similar effects when comparing subjects in mid puberty (6 weeks) with middle aged rats (12 months) (Heine et al., 2004). Most recently a new study determined a 94% decrease in neurogenesis between puberty and middle age for male rats. This decrease was almost solely due to a decline in the production of new cells (McDonald and Wojtowicz, 2005). However none of these studies purposely and directly compared proliferation in pubescents with that in young adult rats (2-3 months), nor have any examined the effects in female rats.

SSRI treatment during the juvenile -pubescent period disrupted normal age related changes in another measure of hippocampal plasticity, dendritic spine density (Norrholm & Ouimet , 2000). Chronic treatment with fluoxetine starting on PND 21(animals killed during puberty at the end of fluoxetine treatment or in adulthood after a washout period) caused retardation in CA1 but not dentate gyrus spine densities with levels equivalent to those seen in untreated 21-day-old animals. Fluoxetine animals differed from all controls, suggesting a lasting effect on the development of hippocampal plasticity even accounting for a 21 day drug wash out period. Given that spine density was differently and lastingly altered by SSRI treatment in juvenile- pubescent treated animals we would also expect to see such effects on neurogenesis.

As animals mature the function of monamines such as serotonin change from being trophic organizing signals to neurotransmitters and neuomodulators (Moll et al., 2000). An examination of the developmental time course of this shift found age related increases in pre-synaptic transporter densities in the frontal cortex, and decreases in densities of the brain stem. Specifically, frontal cortex binding of [^{3}H] paroxetine increased from the juvenile period, through puberty and into adulthood and old age. Brain stem binding decreased sharply from the juvenile period to late puberty, and then increased slightly thereafter (Moll et al., 2000). The authors measured pre-synaptic transporter densities under the assumption these changes would represent changes in the density of innervations of projection fields. Given that the dorsal and medial raphe are located in the brain stem, it is possible that this reduction in transporter density could be involved in the pubertal onset of depression. Furthermore connections to the frontal cortex are strengthened from puberty into adulthood (Paus et al., 1999). It is possible that

a deregulation of the increase in pre-synaptic transporter density at this time could also be involved. Taken together these findings further indicate the need to examine the pubescent response to antidepressants.

Antidepressants and adolescence

Given what little is known about the effects of SSRI's on the brain it is not a surprise that there is now a controversy about the effects of SSRI treatment in adolescents. From the literature it is unclear as to whether SSRIs increase the incidence of suicidal thought or behavior in adolescents. Initially there were a number of case studies reporting increased suicidal thought, attempts and general emotional distress associated with application of SSRIs in adolescents (for review see Couzin, 2004). Experimental studies in humans have been contradictory; however a meta analysis of both published and unpublished studies on SSRIs did indicate an increased risk of suicide and suicidal thoughts for adolescents treated with SSRIs (Whittington, et al., 2004). The authors determined that treatment with fluoxetine (Prozac) had the best risk/benefit profile, where as paroxetine (Paxil) had the worst. Most importantly they determined that paroxetine produced no significant decrease in depressive symptoms for adolescents, and an increased risk of an aversive event. A study by Willens, et al. (2003) indicated that a large percentage (74%) of children and adolescents experienced psychiatric adverse events (PAEs) after being prescribed SSRIs, these PAEs were reversed when subjects were taken off the treatment. PAEs varied in type with the largest group complaining of disturbances in mood. There was no association between PAEs and any specific type of antidepressant. However one of the drawbacks of this study was that suicidal ideation

was not included in the listed measures of PAEs. Jick et al., (2004) determined there was no evidence of an increased odds ratio for suicidal ideation or behavior in a study of children and adolescents prescribed fluoxetine, paroxetine, and 2 tricyclics. It should be noted that their study was done entirely from general practitioner's medical records, which would reduce the likelihood that suicidal thoughts would be reported. Even though these studies were unclear and controversial, the evidence was compelling enough that in 2004 the FDA released a public health advisory warning that treatment of adolescents with SSRI antidepressants could lead to suicidal thought and action (FDA statement 2004). It is our hope that by using an animal model we can determine whether these drugs have a different biological effect on pubescent subjects compared to adults. While this study can not address the issues raised in studies of humans, differences in biological experience can be a foundation for future human studies.

Conclusion

As there is a limited literature addressing how SSRIs affect the pubescent brain, it is important to first characterize the impact of these drugs on pubescent animals and compare them to known effects in adults. Here we will determine whether the SSRI fluoxetine (Prozac) increases cell proliferation and neurogenesis in pubescent and adult female rats as it does for adult males. In experiment 1 the effects of fluoxetine treatment on cell proliferation will be compared between sexes at both age points. Additionally hormonal measures will be examined to determine whether fluoxetine treatment alters sex or stress hormones. In experiment 2 the effects of chronic fluoxetine treatment will be examined in both age groups and sexes after a complete cell cycle has passed. This

will allow us to determine whether the effects of fluoxetine affect cells at a different rate in these age groups or sexes. Experiment 3 will examine the effects of chronic fluoxetine treatment on neurogenesis. Subjects will be treated with fluoxetine during puberty or in adulthood and then sacrificed 28 days later. This will allow us to measure the number of cells that become neurons, and verify cell fate using double labeling for BrdU and neuronal nuclear antigen (NeuN) a marker expressed by adult neurons. Additionally this study will also examine whether there are any effects of fluoxetine treatment on cell fate in adult females and pubescent rats. Finally hormonal measures will also be examined at this time point to determine whether fluoxetine treatment during puberty has lasting developmental effects. Together these studies will provide basic information about the effects of antidepressant therapy on cellular and hormonal function at a time when animals become sexually mature and sex differences in depression emerge.

METHODS

Subjects

Experiments were approved by the Rutgers University Animal Care and Facilities Committee and all work was in compliance with the rules and regulations set out by the Public Health Service policy on the Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals. Pubescent (24-26 days at injection onset) and adult (>60 days <90 days at injection onset) Sprague-Dawley rats were bred on premises from stock obtained from Harlan Sprague-Dawley (Indianapolis, IN). Pubescent rats were weaned between PND 21- 23 and housed individually in the Department of Psychology animal facility at Rutgers University. Adult rats were weaned

between PND 26 -28 and group housed until 60 days of age, at which time they were individually housed. All rats were given *ad libitum* access to water and laboratory chow, and maintained on a 12:12 light/ dark cycle with light onset at 8 am.

For experiment 1 to determine the effects of fluoxetine treatment at a 2 hour proliferation time point the following numbers of subjects were used: pubescent subjects: male/ fluoxetine, n= 8, male/ saline n=15; female/ fluoxetine-diestrus/ proestrus n=7, female / fluoxetine-estrus=8; female/ saline- diestrus/proestrus= 7, female saline-estrus=7. Adults: male/ fluoxetine=14, male/ saline= 13; female/ fluoxetine proestrus= 12, female fluoxetine estrus=7, female/ fluoxetine diestrus=8; female/ saline proestrus= 7, female saline estrus=8, female saline diestrus=8. A subset of these subjects were also used for hormone assays. To determine corticosterone levels 1 day after treatment n=90 (5 per group + 20 naïve animals). To determine estradiol levels 1 day after treatment only females were examined, n=60 (6 per group). To determine testosterone levels 1 day after treatment n=43 (6 per group males; 5 per group of females, no analysis of estrous cycle). For experiment 2 to determine the effects of fluoxetine treatment at a 24 hr proliferation time point the following number of subjects were used: pubescent subjects: male/ fluoxetine n= 6, male/ saline, n=7; female/ fluoxetine n=6, female/ saline=7. Adults: male/ fluoxetine= 9, male/ saline=11; female/ fluoxetine= 8; female/ saline=8. For experiment 3 to determine the effects of fluoxetine treatment on neurogenesis 28 days after BrdU injection the following number of subjects were used: pubescent subjects- male/ fluoxetine n= 9, male/ saline, n=9; female/ fluoxetine n= 7, female/ saline= 8.Adults- male/ fluoxetine= 8, male/ saline=8 ; female/ fluoxetine= 7, female/ saline=8. A subset of the subjects in experiment 3 were also used for double labeling n=32 (four per

group). In addition a subset of these subjects were also used for hormonal assays. To determine corticosterone levels after 29 day washout period n= 40 (5 per group). To determine estradiol levels after a 29 day washout period only female subjects were used, n=24 (6 per group). To determine testosterone levels after a 29 day washout period n= 48 (7 per group males; 5 per group females).

Injections

Subjects received injections (intraperitoneal) of the selective serotonin reuptake inhibitor (SSRI) fluoxetine (5mg/kg) or a weight based equivalent dose of saline vehicle (0.9%) for a minimum of 14 days and a maximum of 18 days. One day after the final fluoxetine/ saline injection subjects received a single injection (intraperitoneal) of Bromo-deoxyuridine (BrdU 200mg/kg) a thymidine analog which is incorporated into the DNA of cells during the S phase of the cell cycle (Miller & Nowakowski, 1988; Cameron and Mckay, 2001). Injections of BrdU were given between 11 am and 2:30pm. Subjects were sacrificed in experiment 1 two hours after BrdU injection; 24 hours after BrdU injection for experiment 2 and 28 days after BrdU injection for experiment 3 (Fig. 1).

Vaginal cytology

During the second week of injections, vaginal swabs were taken daily to assess the stages of the estrous cycle. Using cotton Q-tips immersed in saline, cells were removed from the vaginal track and placed onto slides and subsequently stained with 1% Toluidine blue. The stages of the estrous cycle were verified by visualization under 10X magnification and characterized as follows: Estrus- large blue staining cornified cells,

diestrus - small dark staining leukocytes with scattered epithelial cells, proestrus- round clumped nucleated purple stained cells.

In experiment 1 adult female rats were injected with BrdU and sacrificed at all stages of the estrous cycle; proestrus, estrus, diestrus. Some adult females received more than 14 days (but no more than 18 days) of injections so that all stages of the cycle could be examined. In experiment 2 adult females were in the proestrus stage of the cycle during BrdU injection. Some females received more than 14 days of fluoxetine/ saline treatment with a maximum of 18 days of injection. In experiment 3 vaginal cytology was tracked but no attempt was made to focus on a set stage of the cycle, all subjects received 14 days of injections.

Pubescent rats display an irregular cycle that alternates between a clear stage of estrus and a stage with characteristics of proestrus (large clumps of nucleated cells) and diestrus (leukocytes). Estradiol and progesterone do not fluctuate between these two stages (Hodes & Shors, 2005). In experiment 1 pubescent females were injected with BrdU and sacrificed in both stages of their estrous cycle. For experiments 2 and 3 the estrous cycle was tracked for pubescent females but no attempt was made to use a set stage of cycle for pubescent females.

Sacrifice and perfusion

Subjects were deeply anesthetized with sodium pentobarbital (0.25 ml/kg) and transcardially perfused with 0.9% saline (PH=7.3) followed by 4% paraformaldehyde in a 0.1M phosphate buffer (PH=7.3). Brains were dissected from the skulls, postfixed in 4%

paraformaldehyde for a minimum of 48 hr, and then transferred to PBS (PH=7.3). All subjects were sacrificed between the hours of 1pm and 4:30pm.

BrdU peroxidase staining and immunofluorescent labeling

Coronal sections (40 μm) from the entire rostrocaudal extent of the dentate gyrus were cut from a single hemisphere on a vibratome in a bath of distilled water and 0.1M PBS (pH 7.4). Every 12th slice was mounted in groups of 10-12 per slide (Superfrost plus), dried, and processed for BrdU using peroxidase methods. Brain tissue was heated in 0.1 M citric acid (pH 6.0), rinsed in 0.1M PBS (pH 7.4), and incubated in trypsin for 10 min. Slides were rinsed again, denatured in 2M HCL: PBS for 30 min, rinsed and incubated overnight in primary mouse anti-BrdU (1:200 Becton Dickinson) and 0.5% Tween 20 (1:200) in PBS while stored at 4 degrees C. The next day the slides were subjected to a series of PBS rinses and incubated for 1 hr in biotinylated antimouse antibody (1:200). After another series of rinses the sections were then incubated in avidin–biotin– horseradish peroxidase for 1 hour, and then stained with diaminobenzidine for 7 min. After rinsing in PBS, slides were counterstained with cresyl violet, dehydrated in a series of alcohol rinses, cleared with Xylene and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ)

For double labeling of BrdU and neuronal nuclear antigen (NeuN) free floating single sections were rinsed with 0.1 M TBS (pH=7.5) and denatured in 2 M HCL: TBS for 30 min. Sections were then rinsed and incubated for 2 days with rat anti-BrdU (1:200 with 0.5% Tween 20; Accurate Chemicals, Westbury , NY) plus mouse anti-NeuN (1:500, Chemicon, Temecula, CA) in TBS. Sections were then rinsed and incubated with

biotinylated anti- rat (1:250; Chemicon) in TBS for 90min, rinsed again and incubated for 30 min in the dark with streptavidin-conjugated Alexa 568(1:1000; Invitrogen, Carlsbad, CA) to visualize BrdU and anti-mouse Alexa 488 (1:500; Invitrogen) in TBS to visualize NeuN. Sections were given a final rinse, dried for a minimum of 1 hr and coverslipped using glycerol in TBS (3:1).

Data collection: Microscopy

Slides were coded prior to quantitative analysis and all cell counts were conducted blind to the experimental conditions. For peroxidase stained tissue, cells were visualized under 1000x (100x oil immersion objective with a 10x ocular) magnification on a Nikon eclipse E400 light microscope (Nikon, Melville, New York). Counts were recorded for the number of BrdU labeled cells on every 12th section in a single hemisphere of the entire rostrocaudal extent of the dentate gyrus. The number of BrdU labeled cells were estimated using a modified unbiased stereology protocol that had previously been reported to successfully quantify BrdU labeling (West et al., 1991; Gould et al., 1999). Cell counts were obtained for the combined subgranular zone (SGZ) and granule cell layer (GCL) of every 12th unilateral section avoiding cells in the outermost focal plane. The number of counted cells was than multiplied by 24 (number of intervening slices x number of hemispheres) to give an estimate of the total number of BrdU labeled cells in the dentate gyrus. Only slides with between 8 and 10 countable sections were included for analysis.

For immunofluorescent labeling, all BrdU labeled cells in the SGZ and GL of every 12th unilateral section were scanned and recorded using a Zeiss (Oberkochen,

Germany) LSM 510 confocal laser scanning microscope. Six sections per subject were examined and cells were analyzed using a Plan-Neofluar 40x water immersion objective and dual channel excitation with argon (488nm) and helium-neon (543nm). Co-localization of labeling was determined by obtaining 1 μm thick sections through the optical stack and verification was performed through examination of cells in the orthogonal planes.

Radioimmunoassay

Cardiac blood was collected at the time of sacrifice (1pm- 4:30pm). Blood was collected in with heparin (0.01ml) and centrifuged for 20 min at 3,000 rpm. Plasma aliquots were stored frozen until analysis. Circulating levels of corticosterone, estradiol, and testosterone were analyzed using a solid-phase radioimmunoassay (RIA) system (Coat-A-Count, Diagnostic Products, Corp.) Assay sensitivities for corticosterone, estradiol and testosterone were 5.7ng/ml, 8 pg/ml, 4ng/ dl respectively. Intra-assay variabilities for corticosterone, estradiol and testosterone were 4.3%, 7.0%, 12% respectively. Inter-assay variabilities for Corticosterone, estradiol and testosterone were 5.8%, 8.1%, 12%, respectively

Statistical analysis

Analyses were performed using the statistical program Statistica (StatSoft Inc, Tulsa, OK). Separate 3 factor ANOVAs were performed for pubescent and adult subjects on the number of BrdU labeled cells with sex and treatment (fluoxetine/ saline) as the independent factors. Separate 3 factor ANOVAs were performed on cell counts at the 2

hr time point for pubescent and adult females to determine whether fluoxetine treatment altered proliferation across the estrous cycle. For analyses of percentages of double labeled cells, percentages were converted into arcsin values to remove the fixed limits imposed by percentages that violate the assumptions of parametric statistics. After the data was transformed the arcsin values were analyzed with ANOVA as previously described. Age differences were examined in saline treated controls using 3 factor ANOVA, in which age and sex were the independent factors and the number of BrdU labeled cells was the dependent measure. Separate 3 factor ANOVAs were performed for pubescent and adult subjects to determine the effects of sex and treatment on corticosterone and testosterone levels. In addition adult and pubescent female data were analyzed separately with a 3 factor ANOVA to determine whether fluoxetine treatment altered corticosterone levels in females across the estrous cycle. Pearson's correlations were examined for all hormonal measures and the number of new cells. Separate 3 factor ANOVAs were performed on estradiol concentrations, for pubescent and adult female subjects to examine the effects of the cycle and treatment at a 2hr sacrifice point. Cell counts from pubescent and adult females were analyzed together with a 3 factor ANOVA at a 28 day time point to determine whether age and treatment affected estradiol concentrations. For all ANOVAs Newman-Keuls was used for post-hoc analysis when appropriate. Multiple regression was performed on the number of BrdU labeled cells in the GCL/ SGZ at the combined time points of 2hrs and 24 hrs to determine the effects of treatment, age, sex and time of sacrifice on cell proliferation. A second multiple regression was performed on the number of BrdU labeled cells at the combined time

points of 24 hrs and 28 d to determine the effects of treatment, age, sex and time of sacrifice on cell loss.

RESULTS

Experiment 1- cell proliferation, 2 hr survival

Cell proliferation was measured in subjects sacrificed 2 hrs after BrdU injection. Cells were primarily located at the subgranluar zone (SGZ) in the border between the granule cell layer (GCL) and the hilus (Fig. 2A). ANOVA was used to determine the effects of fluoxetine treatment (fluoxetine/ saline) on cell proliferation in adult females across the stages of the estrous cycle (proestrus, estrus, diestrus)(Fig 5A). There were no interactions between treatment and cycle [$F(2,44)=1.34$, $p>0.05$] and no main effects of treatment [$F(1,44)=1.15$, $p>0.05$] or stage of cycle [$F(2,44)=.25$, $p>0.05$] therefore all adult females were collapsed across cycle and grouped by treatment to compare with males. ANOVA comparing the effects of fluoxetine treatment on cell proliferation in adult male and female rats determined a significant interaction of sex and treatment [$F(1,73)=4.55$, $p<0.05$] (Fig 4A/ Table 1). Post-hoc analysis with Newman-Keuls indicated that fluoxetine treatment increased cell proliferation in adult males compared to their saline counterparts and females of both treatment conditions. Cell proliferation in saline treated males did not differ from females of either condition ($p>0.05$), nor was there a significant effect of fluoxetine treatment on cell proliferation in adult females ($p>0.05$). In addition to the interaction there was a main effect of sex [$F(1, 73)=4.13$, $p<0.05$] indicating that males had higher levels of cell proliferation than females. There

was also a main effect of treatment [$F(1,73)=14.13$, $p<0.05$], fluoxetine treated animals had higher levels of proliferation than saline treated animals.

ANOVA determined that pubescent females had no interactions between treatment and stages of the estrous cycle [$F(1,25)=1.50$, $p>0.05$] nor was there a main effect of treatment [F (1 ,25)=0.42, $p>0.05$] or stage of cycle [F (1,25)=0.01, $p>0.05$] (Fig.5B). Therefore data from pubescent females were collapsed across the stages of cycle to compare with pubescent males using a 3 factor ANOVA. Fluoxetine treatment did not interact with sex during puberty to alter the number of new cells [F (1,48)=0.05, $p>0.05$]. Additionally there were no sex differences [F (1,48)=0.17,$p>0.05$] or effects of chronic fluoxetine treatment [F (1,48)=0.40, $p>0.05$] on cell counts at the 2 hr proliferation time point (Fig. 4B/ Table 1). A 3 factor ANOVA examining the effects of age and sex on the number of BrdU labeled cells in saline treated animals determined that pubescent rats had higher levels of cell proliferation than adults of both sexes [F (1, 61)=57.37, $p<0.05$]. There were no significant interactions between sex and age [F (1,61)=0.009, $p>0.05$] and no main effects of sex [F (1,61)=0.025, $p>0.05$] (Fig. 8A).

Experiment 2- cell proliferation, 24 hrs survival

A three-factor ANOVA was used to examine the effects of fluoxetine treatment in males and females on cell proliferation at a 24 hour survival point for two separately analyzed ages (puberty and adult). Cells at 24 hours generally appeared in clusters or dyads indicating that mitotic division had occurred (Fig. 2B). ANOVA determined that in adult subjects there was a significant interaction between treatment (fluoxetine/saline) and sex (male/ female) on the number of new cells at a 24 hr proliferation time point

[$F(1, 32)=529$, $p<0.05$] (Fig. 4C/ Table 1). Post hoc analysis with Newman-Keuls indicated that fluoxetine treatment increased cell proliferation in adult males compared to all other groups (p values <0.05). Fluoxetine treatment did not significantly increase cell proliferation in adult females ($p > 0.05$). A separate three-factor ANOVA for pubescent subjects indicated that there were no significant interactions between sex or treatment on the number of new cells [$F(1, 22)=3.07$, $p>0.05$]. Furthermore there were no significant main effects of treatment [F (1, 22)=0.84, $p>0.05$] or sex [F (1,22)=0.14, $p>0.05$] (Fig. 4D/ Table 1).

A 3 factor ANOVA was used to analyze age differences in male and female saline treated rats at a 24 hr proliferation time point. Pubescent rats produced more new cells than adults [F (1,29)= 87.10, $p<0.05$] but sex did not affect cell counts [$F(1,29= 2.11$, $p>0.05$] and there were no interactions between sex and age [$F(1,29)=1.40$, $p>0.05$] (Fig. 8B)

Experiment 3-neurogenesis, 28 d survival

A three factor ANOVA was used to examine the effects of fluoxetine treatment in males and females on neurogenesis at a 28 day survival point for two separately analyzed ages (puberty and adult). BrdU labeled cells 28 days after injection were localized in the GCL, had a round shape and generally displayed punctuate staining (Fig. 2C). In adult rats there was a significant interaction between treatment and sex on the number of BrdU peroxidase labeled cells [$F(1, 28)= 4.33$, $p<0.05$] (fig 4E/ Table 1). Post hoc analysis with Newman-Keuls determined that fluoxetine treated males had higher cell counts than saline treated males and females of both conditions (p values <0.05). Saline treated males

also had higher cell counts 28 days after BrdU injection than females of either treatment condition (p values <0.05). Fluoxetine treatment did not alter the number of new cells in adult females (p>0.05).

A subset of the subjects that underwent BrdU labeling with peroxidase methods were also examined with immunofluorescent labeling. The number of BrdU immunofluorescent labeled cells in the SGZ and GCL were counted and examined for co-labeling with the neuronal marker NeuN. Immunofluorescent cells were round in shape with distinct staining for BrdU labeled in red and co-labeling for NeuN in green (Fig. 3A-D). Analysis of the total number of immunofluorescent BrdU labeled cells in the combined SGZ/ GCL indicated a trend towards a significant interaction of sex and treatment [$F(1,12)=3.70$, $p=.08$]. Post-hoc analysis with Newman Keuls determined that fluoxetine treated males had higher numbers of BrdU labeled cells than saline treated males or females of either condition (p values <0.05). Saline treated males did not differ from females (p values >0.05) and fluoxetine treatment did not alter the number of BrdU labeled cells in adult females (p values >0.05). There was a main effect of treatment [$F(1,12)=8.09$, $p<0.05$], fluoxetine increased the number of new cells. There was also a main effect of sex [$F(1,12)=22.46$, $p<0.05$] males had more BrdU labeled cells than females. Analysis of cells that were co-labeled for BrdU and NeuN also indicated that fluoxetine treated adult males had higher levels of new neurons than saline treated males and females of either condition (p values <0.05) (Fig. 6A), whereas there were no significant differences between saline treated males and females of either condition, (p values >0.05) and treatment did not increase the number of new cells in females (p values >0.05). There was no interaction between sex and treatment on the number of double

labeled cells [F (1,12)=1.42, p>0.05]. There was a significant main effects of treatment on the number of double labeled cells [F (1,12)= 6.56, p<0.05], fluoxetine increased the number of new neurons. There was also a main effect of sex [F(1,12)=13.21, p<0.05], males had more new neurons than females. To perform ANOVA on the percentage of double labeled cells, percentages were transformed into arcsin values to remove limits imposed by percentages. Cell fate was not altered by treatment and/ or sex [F(1, 12)=0. 549, p>0.05], nor were there main effects of treatment [F(1,12)=0.946, p>0.05] or sex [F(1,12)=0.094, p>0.05] on the arcsin values (Fig 7A). Approximately 78% $\pm$ 9.7% of the new cells were co-labeled with a neuronal marker.

During puberty a 3 factor ANOVA indicated no interactions between sex and treatment on the number of BrdU peroxidase labeled cells at a 28 day time point [F(1,29)=2.51, p>0.5]. Additionally the number of cells were not affected by treatment [F(1,29)=0.26, p>0.05] or sex [F(1,29)=1.17, p>0.05] (Fig 4F/ Table 1). Examination of a subset of these subjects with an immunofluorescent marker for BrdU also indicated that treatment and sex did not alter the number of cells in the combined SGZ/ GCL [F(1,12)=0.07, p>0.05], nor were there main effects of treatment [F(1,12)=0.00, p>0.05] or sex [F(1,12)=0.60, p>0.05]. Analysis of cells co-labeled for BrdU and NeuN determined there were no interactions between treatment and sex [F(1,12)=0.16, p>0.05] and no main effects of treatment [F(1,12)= 0.2, p>0.05] or sex [F(1,12)=1.13, p>0.05] (Fig 6B). To perform ANOVA on the percentage of double labeled cells in pubescent rats, percentages were transformed into arcsin values to remove limits imposed by using a percentage. Cell fate was not altered by treatment and/or sex in pubescent rats as the arcsin value of double labeled cells did not differ for these groups [F(1, 12)=0.540,

$p>0.05$] and there were no main effects of treatment [$F(1,12)=0.006$, $p>0.05$] or sex [$F(1,12)=1.07$, $p>0.05$] (Fig. 7B). Approximately 80%$\pm$ 9% of the new cells were co-labeled with a neuronal marker.

Cell counts 28 days after BrdU injection were analyzed in saline treated subjects with a 3 factor ANOVA to determine the effects of sex and age on neurogenesis. Pubescent subjects of both sexes had higher numbers of BrdU labeled cells than adults [$F(1,27)=37.71$,$p<0.05$] (Fig 8C), additionally there was a main effect of sex, males had higher numbers of labeled cells than females [$F(1,27)= 17.88$,$p<0.05$]. There were no interactions between sex and age on the number of BrdU labeled cells 28 days after BrdU injection [$F(1,27)= 0.026$, $p>0.05$]. Analysis of immunofluorescent labeled cells in a subset of subjects determined a trend towards an effect of age on the total number of BrdU labeled cell [$F(1,12)=3.25$, $p=.09$] and an effect of age on cells that were double labeled with BrdU and NeuN [$F(1,12)=4.46$, $p=0.056$] suggesting that pubescent subjects had more new neurons than adults. There were no main effects of sex on double labeled cells [$F(1,12)=3.09$, $p>0.05$] and no interactions between age and sex[$F(1,12=0.09$, $p>0.05$]. There were no main effects of sex on total number of BrdU- immunofluorescent labeled cells [$F(1,12)=1.67$, $p>0.05$] or interactions between age and sex [$F(1,12)=0.001$, $p>0.05$].

Cell gain/ loss

To determine the amount of cell gain and the factors affecting it, multiple regression was performed to compare the effects of treatment sex, age and sacrifice time point on combined cell proliferation at 2hrs and 24 hrs (Table 2). There was a significant

liner gain (p<0.05) of 1966± 152 cells in the GCL/ SGZ between time points. Controlling for the other factors pubescent subjects produced 1925 ± 145 more new cells than adults (p <0.05) when cell counts were combine across sacrificial time points. Fluoxetine treatment had a small but significant effect on cell proliferation (p <0.05) in that fluoxetine treated subjects produced 431 ± 143 more new cells than saline treated subjects. Sex did not significantly affect cell proliferation (p>0.05).To determine the amount of cell loss and the factors affecting it a multiple regression was performed comparing the effects of treatment, sex and age and sacrificial time point on cell counts at 24 hrs and 28 days after BrdU injection (Table 3). There was a significant liner decrease (p<0.05) of 1046.15± 180 cells between these two time points**.** Controlling for the other factors pubescent subjects still produced 2217±181 more new cells when time points were combined (p<0.05). In addition males produced 428 ± 179 more new cells than females when time points were combined. Fluoxetine treatment did not significantly affect cell loss when time points were combined (p>0.05).

Hormone concentrations

In a subset of subjects sacrificed 2hr after BrdU injection, corticosterone levels were analyzed to determine whether fluoxetine treatment altered hormonal concentrations. A 3 factor ANOVA was performed on corticosterone concentrations in adult females to determine whether fluoxetine treatment interacted with the estrous cycle to alter circulating levels of corticosterone. There were no interactions between treatment and stage of the estrous cycle on corticosterone concentrations [F(2,24)=0.1, p>0.05]. There were no main effects of treatment [F(1, 24)= 0.59, p>0.05] or stage of

cycle[$F(2,24)=0.56$, $p>0.05$] therefore data for adult females were collapsed across the estrous cycle for comparison with males. ANOVA was performed to determine the effects of sex and treatment on basal corticosterone levels in adult male and female rats. Fluoxetine treatment did not alter basal levels of corticosterone in adult male and female rats [$F(1,36)=0.38$,$p>0.05$] there were no effects of fluoxetine treatment [$F(1,36)=0.35$, $p>0.05$] and no effects of sex [$F(1,36)=0.79$, $p>0.05$] (Fig. 9A). ANOVA was performed on pubescent female rats to determine whether chronic fluoxetine treatment interacted with the stages of the estrous cycle to alter corticosterone levels. There were no interactions between treatment and stage of cycle in pubescent females [$F(1,16)= 0.86$, $p>0.05$] nor were there main effects of treatment [$F(1,16)=0.06$, $p>0.05$] or estrous cycle [$F(1,16)= 0.31$, $p>0.05$] therefore data from pubescent females were collapsed across stage of cycle for comparison with males. A 3 factor ANOVA examining the effects of sex and treatment on corticosterone levels during puberty indicated that there were no interactions between fluoxetine treatment in males and females [$F(1,26)=0.04$, $p>0.05$] and no main effect of fluoxetine treatment [$F(1,26)=0.17$, $p>0.05$]. There was a main effect of sex [$F(1,26)=7.08$, $p<0.05$] pubescent male rats had higher levels of corticosterone than pubescent females (Fig. 9B). Examination of corticosterone levels in animals that had undergone an injection (saline and fluoxetine combined) compared to naïve controls indicated that injection increased corticosterone concentrations in males [$F(1, 26)=6.23$, $p<0.05$], but not in females [$F(1,56)=0.29$, $p>0.05$] when the sexes were examined separately. There were no significant correlations between corticosterone and cell proliferation for any of the groups (p values >0.05).

Circulating hormonal concentrations of corticosterone were also examined in a subset of subjects sacrificed after a 29 day washout period, to determine whether fluoxetine treatment during puberty had a lasting effect on hormonal function in adulthood. Fluoxetine treatment during adulthood did not interact with sex to alter corticosterone levels 29 days after treatment [$F(1,16)=0.10$, $p>0.05$]. There were no main effects of treatment [$F(1,16)=1.23$, $p>0.05$] or sex [$F(1,16)=0.148$, $p>0.05$] (Fig.9C). Treatment with fluoxetine during puberty significantly decreased subsequent adult corticosterone levels in females but not males [$F(1,16)= 7.05$, $p<0.05$] (Fig. 9D). There was a main effect of treatment [$F(1,16)=7.17$, $p<0.05$], fluoxetine treatment decreased in corticosterone levels in pubescent rats but there was no main effect of sex [$F(1,16)=0.005$, $p>0.05$]. There were no correlations between corticosterone levels and neurogenesis for any of the groups.

Estradiol concentrations were analyzed from fluoxetine treated adult and pubescent female subjects at all stages of the estrous cycle sacrificed 1 day after the cessation of treatment. A 3 factor ANOVA indicated that fluoxetine treatment did not interact with the stage of the estrous cycle to alter circulating levels of estradiol [$F(2, 30)= 0.50$, $p>0.05$], nor was there an effect of fluoxetine treatment when data was collapsed across cycle [$F(1, 30)=0.00$, $p>0.05$]. There was a significant main effect of stage of cycle [$F(2, 30=8.36$, $p<0.05$] adult females in proestrus had higher estradiol concentrations than females estrus and diestrus (p values <0.05) (Fig. 10A). Estradiol concentrations did not significantly vary between the other stages of the cycle ($p >0.05$). During puberty fluoxetine treatment interacted with stage of cycle to alter estradiol concentrations [$F(1,20)=4.37$, $p<0.05$] (Fig. 10B). Post hoc analysis with Newman-Keuls

failed to find significant differences and subsequent post-hoc analysis with Fisher's least significant difference test indicated that fluoxetine treatment decreased estradiol concentrations during the estrus stage of the cycle ($p<0.05$). There were no significant main effects of treatment [$F(1,20)= 1.66$, $p>0.05$] or stage of cycle [$F(1,20)= 0.00$, $p>0.05$]. There were no correlations between estradiol concentrations and cell proliferation for any of the conditions (p values >0.05).

A subset of adult and pubescent females were examined after a 29 day wash out period to determine whether there were lasting age and treatment related changes in estradiol concentrations. There was an interaction between treatment and age [$F(1,20)=4.30$, $p=0.05$] (Fig. 10C). Post-hoc analysis with Newman-Keuls indicated that females treated with saline during adulthood had higher levels of estradiol 29 days after cessation of treatment. However as stage of cycle was not examined prior to sacrifice, the possibility that a majority of these females were in proestrus can not be ruled out. There was a trend towards an effect of treatment [$F(1, 20=3.61$, $p=0.07$] but no effect of age [$F(1,20)=2.68$, $p>0.05$].There were no significant correlations when all groups were examined together [$r(22)=-0.30$, $p>0.05$].

Separate 3 factor ANOVAs were performed on adult and pubescent rats to determine whether fluoxetine treatment interacted with sex to alter testosterone levels 1 day after the cessation of treatment. Adult male subjects had higher levels of testosterone than adult females when testosterone levels were analyzed 1 day after cessation of treatment [$F(1,18)=12.14$, $p<0.05$] (Fig. 11A). Treatment with fluoxetine did not interact with sex [$F(1,18)= 0.07$, $p>0.05$] nor was there an effect of treatment when data was collapsed across the sexes [$F(1,18)=0.07$, $p>0.05$]. Effects were similar in pubescent

subjects; males had higher levels of testosterone than females [$F(1,17)=5.43$, $p<0.05$)] (Fig.11B), but there was no interaction between sex and treatment [$F(1, 17)=0.11$, $p>0.05$] and no effect of fluoxetine alone [$F(1,17)=0.00$, $p>0.05$]. There was a significant moderate negative correlation between testosterone levels and cell proliferation when all subjects were examined [r (41)= -0.45, $p<0.05$]. Separate examination of the sexes indicated a negative relationship for testosterone and cell proliferation in males[$r(21)= -0.67$, $p<0.05$] but not females [$r(18)=0.26$, $p>0.05$].

Separate 3 factor ANOVAs were performed on pubescent and adult rats to determine if there were lasting effects of fluoxetine treatment and sex on circulating levels of testosterone after a 29 day wash out period. Adult males had higher levels of testosterone than adult females [$F(1,20)=38.18$, $p<0.05$] regardless of fluoxetine treatment (Fig. 11C). Treatment did not interact with sex to affect testosterone levels [$F(1,20)= 0.76$, $p>0.05$] nor was there a main effect of treatment [$F(1,20)=0.82$, $p>0.05$]. Male subjects treated with fluoxetine and saline during puberty had higher levels of testosterone than their female counterparts in adulthood [$F(1,20)=34.97$, $p<0.05$] (Fig. 11D). There were no effects of treatment on testosterone concentrations [$F(1,20)=1.75$, $p>0.05$] and no interactions between treatment and sex [$F(1,20)1.84$, $p>0.05$]. There were no significant correlations between testosterone levels and neurogenesis for any of the groups examined.

DISCUSSION

Effects of fluoxetine on cell proliferation and neurogenesis

Chronic treatment with antidepressants increases the proliferation of new neurons in the dentate gyrus of the adult hippocampus (Encinas et al., 2006; Malberg et al., 2000).

The effects of this treatment on neurogenesis during puberty have not been examined. This is an important issue because young adults are prescribed antidepressants while they are experiencing the hormonal, anatomical and behavioral changes that accompany adolescence and may interact with drug efficacy. To determine whether chronic antidepressant treatment increased neurogenesis in the pubescent hippocampus, pubescent and adult male and female rats were treated with fluoxetine or saline for 14 days and injected with BrdU on day 15. Subjects were then sacrificed at 3 different time points. One group of animals was sacrificed two hours after the injection of BrdU. This was done in order to assess the number of cells that would potentially divide from a single cohort of cells in the s phase of the cell cycle, presumably without migration or cell death (Nowakowski et al., 1989). A second group was sacrificed one day (24h) after the injection of BrdU. This was done in order to assess the number of cells that divided from the first cohort that had been labeled 24 hrs before (Cameron & Mckay, 2001). Yet another group was sacrificed 28 days after the BrdU injection because it takes at least 10 days for cells to acquire characteristics of neurons and 20 or more days to acquire those associated with mature neurons (Dayer et al., 2003). This time point was used to assess how many of the new cells differentiated into neurons after treatment with the antidepressant. To verify that the cells had indeed differentiated into neurons and to establish the percentage that did so, we also labeled cells with a marker of mature neurons- NeuN. The number of cells that expressed BrdU and NeuN were determined for this purpose.

Chronic treatment with the antidepressant fluoxetine increased the number of new cells in the dentate gyrus of the hippocampus in adult male rats 2 hrs after the BrdU

injection, as well as 24 hrs and 28 days later. There was also an increase in the number of cells that possessed the marker of neuronal maturity, NeuN, indicating that the number of new neurons was increased and was therefore similar to what has been reported (Encinas et al., 2006; Malberg et al., 2000). Chronic treatment with fluoxetine did not affect cell proliferation or neurogenesis in animals that were sacrificed during puberty. There was no effect in females during puberty regardless of the stage of estrus that they were in at the time of BrdU injection. Although fluoxetine did not increase cell proliferation during puberty, the pubescent animals produced many more new cells than did the adults. Thus, the fact that fluoxetine did not increase cell proliferation may reflect an upper limit on the number of new cells that can be produced in response to pharmaceuticals that are used to treat depression.

As noted above, chronic treatment with fluoxetine did increase the number of new cells in adult male rats. However, the increase was only evident in adult males and was not observed in adult females. There was no effect of antidepressant treatment on cell proliferation in adult females during any of the three stages of estrus that we assessed, i.e. proestrus, estrus and diestrus. Others have reported that cell proliferation is increased in response to estrogen in ovariectomized females as well as during proestrus in intact females, when estrogen levels are elevated (Tanapat et al., 1999; 2005). In the present experiments, the increase in estradiol levels during proestrus was not accompanied by an increase in cell proliferation, as determined by cells generated within 2 hrs of the injection and therefore during the cycle in question. It is noted that not all studies have reported increases in cell proliferation during proestrus (Lagrace et al., 2007; Shors et al., 2007). Differences in these studies may reflect differences in the levels of ovarian

hormones during BrdU labeling. Animals in the Tanapat et al., (1999; 2005) study were sacrificed towards the end of the proestrus stage of the cycle whereas subjects in this study were sacrificed during the middle of the proestrus stage of the cycle. Therefore subjects in this study may have been exposed to BrdU labeling while estrogen and progesterone levels were still rising, rather than when they had already reached high levels. This is reflected by the relatively low levels of circulating estrogen reported during proestrus in the current study.

The vast majority of cells in all groups did differentiate into neurons, irrespective of the treatment protocol. The number of cells that were labeled with NeuN was determined with immunofluorescent methods and supported the data using peroxidase methods. Twenty-eight days after BrdU injection the majority of the new cells in all subjects displayed a neuronal marker (78%$\pm$ 9% adults; 80% $\pm$ 9% pubescents). Cell fate was not altered by fluoxetine treatment in any of the groups. This is in agreement with previous research in adult male rats; the ratio of cells displaying neuronal markers to glia was 75%: 13% in one study with an additional 12% not displaying either phenotype (Malberg et al., 2000). A second study indicated that 92% of cells displayed neuronal phenotypes (Encinas et al., 2006). Both the number of new cells and those expressing co-labeling with NeuN were increased in fluoxetine treated adult males, however treatment did not alter neurogenesis in adult females or pubescent subjects of either sex. This finding was expected because fluoxetine did not increase the number of cells during proliferation. Together, these data suggest that daily treatment with the most commonly prescribed antidepressant, fluoxetine, has different effects on cell proliferation and neurogenesis in the hippocampus depending on the sex of the animal and its age.

Cell proliferation and loss

Age, sex, and fluoxetine treatment are all factors that may affect cell proliferation, either through combined action or as a separate influence. Therefore we wanted to determine whether each of these factors had a separate effect on cell proliferation. The 2 hr and 24 hr time points are both considered measures of cell proliferation. At 2 hrs BrdU labels a single cohort of cells in the s-phase of the cell cycle (Nowakowski et al., 1989). Therefore these cells have not yet divided and produced daughter cells. At 24 hours cells have gone thorough one complete cell cycle, divided and created progeny (Cameron & Mckay, 2001). To asses how age, sex, treatment and time of sacrifice affected cell proliferation, the cell counts from both sacrificial time points were combined and analyzed. All rats had an increase in cell proliferation from 2 hrs to 24 hrs of approximately 1966 $\pm$ 153 new cells indicating that cells divided and produced progeny between these time points. This is in agreement with previous research which determined a 1.7 fold increase in the number of new cells generated in 24 hrs (Dayer et al., 2003). Age was the next most important factor in affecting cell proliferation. Previous research indicated that pubescent male rats have increased cell proliferation compared to middle aged (12 months) male rats when sacrificed 24 hrs after BrdU injection (Heine, et al., 2004; McDonald & Wojtowicz, 2005). Here we report that both male and female rats have increased cell proliferation when compared to young (60-90 day) adults. Pubescent animals produced on average 1925 $\pm$ 145 more new cells than adults during cell proliferation. Fluoxetine treatment had a small but significant effect on cell proliferation. Fluoxetine treated animals produced on average 431$\pm$ 143 more new cells than saline

treated controls. The sex of the animals did not significantly affect cell proliferation; males produced on average 185±145 more new cells than females. Therefore of the factors examined, the age of the animal and the type of drug treatment separately affected cell proliferation. The age of the subject had a larger effect on cell proliferation than the drug treatment.

New cells are produced in the dentate gyrus of the hippocampus, however not all of the cells produced survive and become incorporated. A study examining cell loss in the adult male rat indicated that from day 6-28 approximately 48 % of the newly generated cells in the granular cell layer died by the end of the time period (Dayer et al, 2003). It is thought that these cells die through apoptosis rather than any form of traumatic death and are part of a cell turnover process (Dayer et al., 2003; Heine et al., 2004).

Given that approximately half of the newly generated cells would die, we wanted to determine how the factors of age, sex and fluoxetine treatment influenced cell survival from cell proliferation at 24 hrs to neurogenesis at 28 days after BrdU injection. In order to determine this, cell counts at 24 hrs and 28 days after BrdU injection were combined and analyzed to determine if age, sex, fluoxetine treatment and time of sacrifice influenced cell survival. There was a decrease in cell counts from 24hrs to 28 days of approximately 1046± 180 cells for all groups. The decrease in cell survival was less than what has previously been reported (3380± 1244) from day 6-28 (Dayer et al, 2003). It is likely that the size difference of the decrease reflects an initial increase in cells from day 1-6 which was not measured in the current study. Even though cells were lost from day 1-28 pubescent rats still had more new cells across these time points than adults. Pubescent

rats produced on average 2217±180 more new cells than adults. Previous research has indicated that pubescent male rats produce more new cells in the SGZ than middle aged (12 month) males but also have increased apoptosis in this area (Heine, et al., 2004). Apoptosis can be measured by DNA fragmentation indicated by terminal transferase-mediated dUTP nick-end- labeling (TUNEL) (Czeh & Lucassen, 2007). While pubescent male rats had a larger number of TUNEL stained cells in the SGZ, rates of apoptosis remained stable from puberty to middle age for all other areas of the dentate gyrus (Heine et al., 2004). Furthermore, levels of cell proliferation were larger in pubescent rats (~5000 new cells) than the rate of cell death (~ 300) supporting the current finding that a smaller proportion of the newly generated cells die during puberty. In addition to the effects of age, sex was also a factor that affected cell survival. Males had on average 428± 180 more new cells than females. Previous research has also suggested that females have increased cell loss compared to males (Tanapat et al., 1999). Freely cycling adult females had a transient increase in cell proliferation compared to males, but there were no differences in neurogenesis when it was measured 2 weeks after BrdU injection, indicating that the females had lost additional cells. Furthermore, female rats at the 2 week time point had increased numbers of pyknotic cells suggesting that there was increased cell death. Fluoxetine treatment was not indicated as a factor in protection against cell loss. This is supported by previous research which indicated that fluoxetine acts to increase cell proliferation, but has no effects on the survival of those cells (Encinas et al., 2006; Malberg et al., 2000).

Effects of fluoxetine on Hormones

The hormones corticosterone, estradiol and testosterone all affect neurogenesis. Adrenalectomy increases neurogenesis and application of exogenous corticosterone decreases neurogenesis in adult male rats (Cameron & Gould, 1994). Ovariectomy in females decreases neurogenesis and application of estradiol restores neurogenesis to pre-surgery levels (Tanapat et al., 1999; 2005; Banasr et al, 2001). Furthermore natural fluctuations in cell proliferation occur across the estrous cycle in female rats, and females have higher levels of cell proliferation when estrogen levels are at their peak (Tanapat et al., 1999). Testosterone does not increase cell proliferation but does affect cell survival (for review see Galea et al., 2006). As these hormones may mediate the effects of fluoxetine on neurogenesis, it was necessary to determine whether fluoxetine altered the circulating levels of these hormones. To determine the effects of fluoxetine treatment on the circulating levels of the hormones corticosterone, estradiol and testosterone, subjects were sacrificed 1 day after the cessation of treatment or 29 days after the cessation of treatment and hormone levels were measured with a solid phase RIA kit. The 1 day time point was used to determine whether fluoxetine had immediate effects on basal hormonal concentrations. The 29 day washout period was used to determine whether fluoxetine had lasting developmental effects on hormonal concentrations.

Fluoxetine treatment (10mg/kg) was reported to increase basal plasma and brain levels of corticosterone in adult male rats (Weber et al., 2006; Serra et al., 2001). However others report no increases in basal or post-stress plasma corticosterone concentrations for adult male rats with a lower dose (4mg/kg)(Stout et al., 2002). Exposure to an acute stressor of 30 min immobilization or 15 min swim stress increased corticosterone concentrations for all rats, but there was no effect of 27 days of treatment

with a variety of SSRIs or tricyclic antidepressants on corticosterone concentrations. In addition a higher dose (10mg/kg) did not affect basal corticosterone levels in female rats (Van de Kar et al., 2002) suggesting that fluoxetine may increase plasma corticosterone concentrations in males but not females at the same dose. Prior to puberty male rats chronically treated with the same dose of fluoxetine (10mg/kg) also did not have increased basal levels of CORT or ACTH (Landry et al., 2005), however the effects of fluoxetine treatment on corticosterone concentrations during puberty are unknown. Therefore we proposed that chronic treatment with fluoxetine would increase basal concentrations of corticosterone in adult males, but not pubescent males or females of either age. There were no effects of fluoxetine treatment on basal corticosterone concentrations in adult males sacrificed 1 day after cessation of treatment. This finding may reflect a dose response difference as our dose (5mg/kg) was more similar to that reported by Stout et al., (2002) (4mg/kg) who did not report an effect of fluoxetine on basal corticosterone concentrations than the dose used by (Weber et al., 2006) (10 mg/kg) who did report an effect. In agreement with the study by Van de Kar et al., (2002) there was no effect of fluoxetine treatment on basal concentrations of corticosterone in adult females one day after treatment. In addition there were no effects of chronic fluoxetine treatment on basal corticosterone concentrations in pubescent rats of either sex one day after treatment. At this time point there was a sex difference in basal corticosterone concentrations during puberty but not during adulthood. Pubescent male rats had higher levels of circulating corticosterone than females regardless of treatment. This may reflect an effect of injection stress as previous research in untreated rats has indicated there is no sex difference in basal concentrations of corticosterone during puberty (Hodes & Shors,

2005). Injected pubescent male rats had increased levels of corticosterone compared to naïve controls regardless of the type of treatment in the current study and the increase may account for the reported sex difference. There was no sex difference in the levels of circulating corticosterone during adulthood. Previous research in untreated rats determined that adult females have higher levels of average daily circulating corticosterone than males during proestrus and diestrus but not estrus (Atkinson & Waddell, 1997). The lack of a sex difference in adult animals in the current study may result from grouping females from all stages of the estrous cycle.

Treatment from PND 4-21 with fluoxetine increased anxiety and depression associated behaviors in mice tested during adulthood (Ansorge et al., 2004). Treatment with fluoxetine during development lead to decreased exploration of the open field, increased anxiety in the elevated plus maze, and increased latencies to escape a shock. This study did not examine hormonal profiles of the mice, but did suggest that treatment with fluoxetine during a critical developmental period may lead to lasting alterations in behaviors that affect corticosterone levels. To determine whether fluoxetine treatment had lasting effects on basal corticosterone levels for subjects treated during puberty, a second group of rats was sacrificed after a 29 day washout period. Chronic treatment with fluoxetine had a lasting effect on pubescent female rats. Female rats treated with fluoxetine during puberty and sacrificed in adulthood after a 29 day washout period had lower levels of corticosterone than their saline treated counterparts or males of either condition. Treatment with fluoxetine during adulthood did not alter corticosterone levels in adult males or females after a 29 day washout period. These data suggest that puberty in the female rather than the male increases susceptibility to the developmental effects of

fluoxetine treatment. In addition these data suggest that females treated with fluoxetine during puberty may have an abnormal stress response in adulthood and this hypothesis requires further testing. However these data suggest a dissociation between the effects of fluoxetine treatment on corticosterone and neurogenesis in the pubescent female as females with decreased corticosterone levels did not have increased neurogenesis. In addition these data suggest that alterations in basal corticosterone levels are not involved in the effects of fluoxetine treatment on neurogenesis for any of the other groups of subjects.

The circulating level of estrogen has been implicated in the etiology of female depression. Depression is twice as likely to occur in women than men (Earls, 1987; Kessler, 2003) This sex difference in the occurrence of depression begins with puberty and ceases with menopause (Katiala-Heino et al., 2003; Marcotte et al., 2002; Piccinelli & Wilkinson, 2000; Ruiz et al., 2000; Sonnenberg et al., 2000) suggesting an activational role for estrogen in female depression. Data from rodent studies are conflicting on whether fluoxetine treatment alters the estrous cycle and may reflect breed differences. Fischer 344 rats injected for 12-16 days with fluoxetine (10 mg/kg) displayed cessation of the estrous cycle or an elongated estrous cycle in addition to decreased sexual responsiveness (Uphouse et al., 2006). A normal cycle reappeared with longer treatment with fluoxetine (16-25 days). Fluoxetine did not decrease estradiol concentrations but did decrease progesterone concentrations. A different study in Sprague-Dawley rats indicated no effects of chronic treatment with fluoxetine (10mg/kg) for 15 days on circulating estrogen levels or length of cycle (Van de Kar et al., 2002). However in ovariectomized female Long-Evans rats given estrogen replacement, chronic treatment with fluoxetine

decreased circulating levels of estrogen in a linear dose dependent manner with higher doses (5mg/kg) decreasing the levels of estrogen less than lower doses (0.5-2mg/kg) (Taylor et al., 2004). Therefore it is unclear whether fluoxetine affects estradiol concentrations in female rats. To determine whether chronic fluoxetine treatment decreased circulating levels of estradiol in pubescent and adult female rats, animals were sacrificed 1 day after the cessation of treatment.

Pubescent females cycle between two stages of estrus, one that resembles diestrus (leukocytes are present) with some characteristics of proestrus (round, nucleated clumped cells), and a clearly defined stage of estrus (large non-nucleated cornified cells). While estradiol levels normally do not fluctuate between these two stages of the cycle (Hodes & Shors, 2005), treatment with fluoxetine decreased estradiol levels during the estrus stage. This response to fluoxetine did not occur in the adults. Therefore, fluoxetine did not effect estradiol concentrations during any of the three stages of the adult cycle (proestrus, estrus, diestrus). There was a significant effect of cycle in adult females regardless of treatment. As expected adult females in proestrus had higher levels of circulating estradiol than female in the other stages of the cycle.

To determine whether chronic treatment with fluoxetine had lasting effects on estradiol levels, pubescent and adult females were sacrificed 29 days after the cessation of treatment. Adult saline treated female rats had higher levels of estradiol than fluoxetine treated adult females and pubescent females of both conditions. Unfortunately the stage of the estrous cycle was not evaluated in these females immediately prior to sacrifice therefore we are unable to dissociate the lasting effects of fluoxetine from normal variations across the estrous cycle. Taken together these data suggest that puberty in the

female is a period of increased susceptibility to the effects of fluoxetine treatment on estradiol concentrations, however these hormonal effects are separate from and do not influence cell proliferation or neurogenesis.

Studies of seasonal breeders suggest that higher levels of testosterone increase cell survival without necessarily increasing cell proliferation (for review see Galea et al., 2006). Male songbirds have increased neurogenesis in the higher vocal center (HVC) when their androgen levels are high but more of these cells die when testosterone levels fall after the breeding season (Alvarez- Buylla & Kirn, 1997). Under natural conditions Female canaries have low levels of neurogenesis in the HVC, however treating female canaries with testosterone caused a 3-fold increase in the number of new cells in the HVC (Rasika et al., 1994). Short term survival (3 days after testosterone replacement/ 1 day after [^{3}H] thymidine injection) did not increase in the number of labeled cells in the areas of the bird brain where new cells are born indicating that there were no effects of testosterone on cell proliferation. Hormone treatment 20 or 40 days after [^{3}H]thymidine labeling did increase the number of new cells suggesting that testosterone rescued more new cells from death (Rasika et al., 1994). Data from rodent seasonal breeders also suggest that testosterone increases cell survival but does not increase cell proliferation (Ormerod & Galea, 2003b). Male meadow voles were exposed to short or long photoperiods to simulate breeding seasons and effects of photo period on breeding status were verified by testes mass. Cell proliferation as measured by the density of BrdU labeled cells 2hr after injection did not differ between short and long photo period animals. However there were more new cells in reproductively viable males (long photo

period) 5 weeks after injection of [^{3}H] thymidine suggesting that more new cells had survived.

Previous research suggested the possibility that testosterone levels may influence the effects of fluoxetine treatment. Female rats as early as PND 28 have higher number of 5HT1A and 2A receptors in the hippocampus. Castration of male rats at the same age increased the number of receptors to female levels, an effect that was prevented by exogenous testosterone administration (Zhang et al., 1999). It should be noted that male rats at this age have low levels of circulating testosterone (15$\pm$ 5 ng/d) compared to adults (190 $\pm$ 37 ng/dl) however levels are still higher than those found in pre-pubescent females (2 $\pm$ 1 ng/ dl) (Hodes& Shors, 2005). A study examining chronic social stress in male rats indicated that high (10 mg/kg) doses of fluoxetine decreased circulating levels of testosterone in both chronically stressed and unstressed rats compared to unstressed controls (Rygula et al., 2006). Low doses of fluoxetine (0.75mg/kg) decreased male sexual and aggressive behavior but did not alter circulating levels of testosterone. (Taylor et al., 1996). To determine whether fluoxetine treatment decreased circulating levels of testosterone, male and female pubescent and adult rats were sacrificed 1 day after the cessation of treatment. To determine whether fluoxetine treatment had lasting effects on testosterone levels, animals were sacrificed 29 days after the cessation of treatment.

Testosterone levels were not altered by fluoxetine treatment in either adult or pubescent males 1 day or 29 days after treatment. As expected, males had higher levels of testosterone than their same aged female counterparts. Chronic treatment with fluoxetine also did not alter testosterone levels in females at either age or sacrificial time point. We attempted to associate individual differences in testosterone concentrations with the

number of new cells that were generated in males. Testosterone levels in males negatively correlated with cell proliferation, the more testosterone the rat had the lower his number of new cells. The age of the male rats at the time of sacrifice may be the basis of this correlation. There was no correlation between testosterone levels and neurogenesis when male rats were sacrificed after a 29 day washout period. Taken together these data indicate that fluoxetine treatment does not alter endogenous levels of testosterone in male or female rats. Furthermore there are no lasting effects of fluoxetine treatment on testosterone levels. There is a relationship between circulating levels of testosterone and cell proliferation as males with low levels of testosterone had increased cell proliferation, but it is likely that an additional factor such as age contributed to both effects.

Fluoxetine treatment and neurogenesis: possible mechanisms

Since the initial report, a number of studies have investigated the mechanisms by which fluoxetine increases cell proliferation. The effects of fluoxetine on the neuronal differentiation cascade was visualized with confocal microscopy and delineated using nestin- GFP transgenic mice (Encinas et al., 2006). Cells with a triangular soma, that had positive labeling for glial fibrillary acidic protein (GFAP positive) and a low rate of cell division were designated as quiescent neural progenitor cells. These cells are considered the most primitive stem-like population in the dentate gyrus, they divide asymmetrically, and only a fraction (>2%) of these cells can be labeled with BrdU. The next class of progenitor cells was classified as amplifying neural progenitors. These cells had a distinct morphology of a small round or oval soma and did not stain positive for GFAP or doublecortin (DCX) an early neuronal marker. A larger proportion of these cells are

labeled with BrdU (20-25% after 2hr BrdU injection). It is thought that quiescent neuronal progenitors give rise to these amplifying neural progenitor cells. The amplifying neuronal progenitors in turn give rise to cells designated as neuroblasts type 1 and eventually type 2. These cells have a larger soma and a horizontal process that extends into the plane of the SGZ. These cells begin to express DCX and other markers of young neurons. They migrate and eventually become the immature neurons located in the SGZ. At the earliest stage a subclass of neuroblasts type 1 have a smaller soma and shorter neuronal process, and morphologically resemble the amplifying neuronal progenitor cells. This subclass is also labeled by BrdU. Analysis of cells labeled with BrdU 24 hrs after injection indicated that fluoxetine treatment increased the symmetrical division of the amplifying neural progenitor cells. In addition fluoxetine treatment also increased the number of BrdU labeled neuroblasts type 1 cells, but did not affect the number of neuroblasts type 2 cells. Fluoxetine treatment did not increase the number of quiescent neuronal progenitors undergoing division. Therefore fluoxetine treatment is thought to increase the number of new cells in the dentate gyrus of the hippocampus by increasing the number of amplifying neuronal progenitors that undergo division as well as increasing the number of their progeny that further undergo division. It should be noted that fluoxetine does not increase the number of new cells in the subventricular zone (SVZ) another area which gives rise to new neurons (Encinas et al., 2006; Malberg et al., 2000). Therefore fluoxetine does not have an overall effect on cell division but instead acts specifically at the dentate gyrus.

Fluoxetine treatment increases the expression of brain derived neurotrophic factor (BDNF) in the cortex and hippocampus (Wyneken et al., 2006). A low dose of fluoxetine

(0.67 mg/kg) increased BDNF content in homogenates of the cortex and hippocampus after 9 days of treatment and levels remained elevated for 6 weeks. It was initially thought that BDNF might be involved in the effects of fluoxetine treatment on neurogenesis in the hippocampus (Duman, 2004b) as BDNF had antidepressant like effects in behavioral models of depression, expression was down-regulated by stress and up-regulated by antidepressant treatment. However, it was determined that BDNF increases the survival of new cells in the hippocampus but does not increase cell proliferation (Sairanen et al., 2005). Transgenic mice that had reduced BDNF levels or impaired trkB activation and their wild type controls were used to determine if antidepressants increased neurogenesis when BDNF levels were low. Chronic fluoxetine and imipramine treatment both increased the number of new cells produced 24 hrs after BrdU injection in all types of mice. Cell counts of wild type mice remained elevated 21 days after antidepressant treatment compared to saline injected controls, but did not differ from saline controls in either form of transgenic mouse. In other words, fewer of the new cells produced by antidepressant treatment survived in transgenic mice. Therefore it is not likely that BDNF expression is involved with the increases in cell proliferation triggered by antidepressant treatment, instead this growth factor is probably acting to keep the new cells alive and allow them to mature and become incorporated into the dentate gyrus.

Serotonin receptors, specifically the 5-HT1A receptor, may be involved in the increase in neurogenesis after chronic antidepressant treatment. There are more than 14 subtypes of 5HT receptors that have been identified and their role in depression and antidepressant treatment still needs to be determined (Lesch, 2001). The 5HT receptors

are g-coupled receptors with the exception of 5HT3 receptors which is a ligand –gated ion channel (for review see Hoyer et al., 2002). The actions of 5HT1 receptors are generally inhibitory through coupling with G proteins to inhibit cAMP formation. The 5HT1 class of receptors are located pre-synaptically as autoreceptors and post-synaptically on a variety of cell types including pyramidal cells in areas CA1/CA3 and granule cells in the dentate gyrus. The 5HT2 class are located post-synaptically and is excitatory. These receptors couple with G proteins to increase hydrolysis of phosphates and elevate cellular calcium. The 5HT3 receptors have excitatory effects and act as ion channels. The 5HT4 , 5HT6 and 5HT 7 class of receptors couple with G proteins to promote cAMP formation. The 5HT5 class of receptors has an unclear physiological functional response but may negatively couple to adenlyte cyclase in astrocytes (Hoyer et al., 2002; Ressler & Nemerofff, 2000). The effects of the subtypes of receptors are various and beyond the scope of this paper. Only the 5HT1A receptor has been determined to affect neurogenesis (Santarelli et al., 2003) and its effects and functions will be discussed in detail.

Pre-synaptic 5HT1A receptors or somatodendritic receptors are located primarily in the dorsal and median raphe. When activated by an agonist these receptors hyperpolarize 5HT cell membranes through the opening of a potassium channels and reduce firing rates (for review see de Vry, 1995). This leads to the suppression of 5HT synthesis, turnover and release in projection areas. Post synaptic 5HT1A receptors are found in limbic areas such as the hippocampus, septum and cortex. Activation of 5HT1A receptors also generally induce membrane hyperpolarization and decrease cell firing. However in some instances activation of 5HT1A receptors facilitate cell firing.

Activation of 5HT1A receptors increased the population spike amplitude of granule cells but did not affect the granular layer population EPSP, suggesting that activation these receptors would increase the likelihood that an action potential would pass from one cell to another, but without increasing baseline activity (Klancnik et al., 1989). Therefore post synaptic 5HT1A receptors have both inhibitory and excitatory effects in different areas of the hippocampus.

Antidepressants act to increase 5HT transmission in the brain; however the exact mechanisms have still not been determined. Selective serotonin re-uptake inhibitors (SSRIs) act immediately on the brain to increase levels of extracellular serotonin (5HT) in areas such as the prefrontal cortex, dorsal striatum and hippocampus (Hervas and Artigas, 2000), however negative feedback through pre-synaptic autoreceptors reduces the amount of 5HT being produced. Blocking these autoreceptors has been shown to potentate the release of 5HT in these areas, and may be a way of shorting the lag time of treatment efficacy. However, depending on the system used (systemic or local administration) there are differences in the amounts released per area. It is most difficult to increase amounts of 5HT in the hippocampus, compared to all other areas (Hervas & Artigas, 2000). Chronic treatment with fluoxetine may act to desensitize 5HT1A autoreceptors. Data from electrophysiology studies indicate that 5HT neurons eventually resume normal baseline rates of firing and the 5HT1A receptors have reduced sensitivity to a 5HT1A receptor challenge (Blier et al., 1987). While pre-synaptic receptors may become desensitized, post- synaptic receptors do not. Chronic treatment with citalopram did not alter 5HT1A sensitivity of hippocampal 5HT1A receptors when dialysate 5HT was measured after an 8-OH-DPAT challenge (Hjorth & Auerbach, 1994). In general it is

thought that pre-synaptic receptors are more sensitive to the actions of agonist than post synaptic receptors and therefore higher doses of drugs may be necessary to alter post-synaptic receptor function (Hjorth et al., 2000).

Antidepressant treatment may act to decrease the number of 5HT1A receptors in the hippocampus (Klimiek et al, 1994; Subhash et al., 2000). This would in turn, increase the activity of hippocampal neurons. The effects of acute and chronic fluoxetine and citalopram treatment differently affected 5HT1A receptor density and binding in adult male rats. Acute treatment with fluoxetine (20 mg/kg) but not citalopram (20mg/kg) decreased the density of receptors in the hippocampus (Klimek, et al., 1994). Chronic treatment with citalopram (10 mg/kg) increased receptor density, whereas fluoxetine (10 mg/kg) treatment did not increase the number of receptors. Chronic fluoxetine treatment did induce an increase in receptor density from that recorded with acute treatment suggesting that fluoxetine initially decreases receptor density, but over time the number of the receptors increase to normal levels. While receptor density changed with antidepressant treatment there were no effects on binding affinity, suggesting that antidepressant treatment did not alter the efficacy of these receptors. In another report chronic treatment with fluoxetine (10mg/kg) did decrease 5HT1A receptor density, but again binding affinity was unaffected (Subhash et al., 2000). However in un-medicated human depressives PET scan examinations indicated lower levels of 5HT1A receptor binding in the hippocampus (Drevets et al., 1999). Therefore the relationship between receptor density, receptor binding affinity and antidepressant treatment remains to be determined.

Chronic activation of 5HT1A receptors with the agonist 8-OH-DPAT increases cell proliferation and are involved in the effects of fluoxetine treatment on cell proliferation (Santarelli et al., 2003; Huang & Herbert, 2005b). The involvement of 5HT1A receptors in the effects of antidepressant treatment was determined using 5HT1A receptor knock out (KO) mice and their wild-type controls (Santarelli et al., 2003). Mice were treated with fluoxetine, imipramine (a tricyclic) or vehicle for 27 days and then injected with BrdU and sacrificed 24 hrs later or 28 days later. Fluoxetine treatment increased the number of BrdU labeled cells at both time points in wild-type but not in 5HT1A receptor KO mice. Imipramine increased cell proliferation and neurogenesis in both wild-types and KOs suggesting that tricyclic antidepressant have a different mechanism of action on neurogenesis. More importantly chronic treatment with 8-OH-DPAT increased cell proliferation in wild type controls, but not KO mice. The increase was of a similar magnitude to the amount reported with antidepressant treatment, suggesting that fluoxetine increases cell proliferation through activation of 5HT1A receptors. Chronic treatment with 8-OH-DPAT in rats also increased cell proliferation, and this effect was blocked by application of the 5HT1A antagonist WAY-100635 (Huang & Herbert, 2005b). In addition manipulations that affect neurogenesis in animal studies affect 5HT1A receptors similarly. In rats glucocorticoids decrease 5HT1A receptor expression (Chalmers et al., 1993) and adrenalectomy increases 5HT1A receptor binding (Mendelson & McEwen, 1992). However the effects of adrenalectomy on cell proliferation are not dependant on the activation of 5HT1A receptors as the antagonist WAY-100635 did not block the effects of adrenalectomy on cell proliferation (Huang & Herbert, 2005b). Therefore while 5HT1A receptors likely mediate the effects of

fluoxetine on neurogenesis their down-regulation may not be involved in stress effects on neurogenesis.

Age differences in fluoxetine treatment possible mechanisms

Chronic treatment with fluoxetine increased cell proliferation and neurogenesis in adult male rats, but not pubescent rats of either sex. This age difference is possibly due to changes in 5HT1A receptor density and binding affinity during maturation. Prior to puberty (30 days) male and female rats display higher levels of 5HT1A mRNA and protein, in the dentate gyrus, and CA1 than adults (90 days) (Stamatakis et al., 2006). More importantly prepubescent male and female rats show a lower affinity of binding for the 5HT1A receptor agonist 8-OH-DPAT suggesting that drugs that are supposed to increase function and possibly neurogenesis in prepubescent subjects have a decreased ability to do so. As age differences in 5HT1A receptor density and affinity may mediate age differences in the effects of fluoxetine treatment on neurogenesis, further experimentation should be conducted to elucidate this relationship

In adult rats chronic treatment with fluoxetine increased cell proliferation and neurogenesis in male rats but not female rats. During puberty there were no sex differences in the effect of fluoxetine treatment or on the number of cells produced by either sex regardless of treatment. Sex differences in the number of new cells during puberty have previously been reported in untreated rats (Perfilieva et al., 2001). Male and female pubescent (5 weeks) rats were injected for 7 days with BrdU (50mg/kg) and sacrificed 24 hrs or 30 days after the last injection. Female rats had fewer BrdU labeled cells than males at 24 hrs but not 30 days after injections ceased, suggesting that the sex

difference was transient and possibly related to age at time of sacrifice. It should also be noted that there are dose dependent effects of BrdU injection in rats at the tested age (5 weeks) (Cameron & Mckay, 2001). In general a larger single dose (200-300mg/kg) is thought to fully saturate the brain and produce more uniform cell counts than the smaller repeated dose (50 mg/kg) used in the above mentioned study (Christie & Cameron, 2006). Therefore full saturation may not have occurred in the pubescent females resulting in the lower cell counts.

Pubescent rats of both sexes produced more new cells than adults. It is possible that cells may be generated at a maximum rate during puberty that cannot be surpassed. While this is possible it is improbable, as two week old rats can generate 30,000 new cells in 24 hours (Heine et al., 2004). To further address this issue it would be helpful to determine whether fluoxetine would in turn increase cell proliferation in animals that had less than the normal degree of cell proliferation. Others have reported that maternal separation during PND 14-21 reduced levels of cell proliferation in male rats sacrificed on PND 21. One week of treatment with fluoxetine increased cell proliferation in the maternally separated rats. The number of BrdU labeled cells did not differ between fluoxetine treated subjects and non-separated controls (Lee et al., 2001). Therefore the possibility exists that during maturation these drugs do not alter cell proliferation, but do block abnormal decreases related to stress.

Drugs are metabolized at different rates depending upon the age of the animal. As rats mature through puberty a sexual dimorphism in the ability of the liver to metabolize drugs appears (El Masry et al., 1974). In adulthood male rats have a 3-5 fold increase in the number of drug metabolizing enzymes in the liver (Shapiro et al., 1995).

The numbers of metabolizing enzymes become sexually dimorphic during puberty as the rate of metabolism increases in males and decreases in females. These changes in drug metabolism have been linked to androgens and growth hormone (El Masry et al., 1974).

The data on age related changes in SSRI metabolism, binding density and affinity are conflicting. Chronic fluoxetine treatment and other SSRIs in neonatal (PND-7 day) and adult rats (3-4 months) indicated no age differences in the amount of binding of [^{3}H] paroxetine. There were also no reported age differences in endogenous serotonin levels or the metabolite 5-hydroxyindoleacetic acid (5HIAA) in the hippocampus or raphe (Dewar et al., 1993). However data from the un-stressed saline treated control group in a study on pre-natal fluoxetine treatment suggest that prepubescent (PND26) rats have higher 5HT turnover than adults (PD 70) (Cabera-Vera et al., 1997). While subjects had similar levels of 5HT in the hippocampus, there were differences in the amounts of 5HIAA and the 5HIAA/5HT ratio indicating that pre-pubescent subjects naturally had higher turnover and increased levels of the metabolites. No age differences were found in the reduction on 5HT when PCPA was administered. This study supports the premise that higher doses of fluoxetine maybe needed for pubescent males to induce the same cellular effects that are seen adults.

The present results indicate that fluoxetine does not increase cell proliferation during puberty. However the possibility exists that other types of SSRI antidepressants or fluoxetine at a higher dose may increase cell proliferation in pubescent rats. Pubescent male rats treated with citalopram, but not the tricyclic desipramine, had increased cell proliferation and increased a coping measure in the modified forced swim test (Hoshaw et al., 2006). Both drugs increased cell proliferation as well as coping behaviors in adult

males supporting the idea that some antidepressants act differently in pubescent and adult males. However in humans fluoxetine is the only SSRI that has been approved for use in adolescents (FDA statement, 2004) and citalopram is one of the drugs that is considered to have a negative risk to benefit profile (Whittington et al., 2004). Citalopram did not reduce depressive symptoms in subjects of 7-18 years of age and was associated with an increase in aversive events. Viewed together these data raise the possibility that a further increase in cell proliferation during puberty may not necessarily be a beneficial outcome.

Fluoxetine treatment did not reduce cell proliferation or neurogenesis during puberty. Examination of another measure of hippocampal plasticity, indicated retardation in spine density development for subjects treated with fluoxetine during pubertal development (Norholm & Ouimet, 2000). Furthermore early life (PND 4-21) treatment with fluoxetine and other antidepressants induce anxiety and depression related behaviors during adulthood (Ansorge et al., 2004; Morelli et al., 2006). Additional work should be done to determine whether treatment during puberty has detrimental effects on behavioral measures of anxiety and depression in adulthood. This is a possibility as puberty is now being considered as a second organizational period of development (Sisk & Zehr, 2005) and many physical changes have been determined in limbic areas implicated in depression (Giedd et al., 1997; Cooke et al., 2007).

While fluoxetine treatment during puberty did not alter a cellular measure of plasticity, it did alter the hormonal profile of females. Fluoxetine treatment decreased circulating levels of estradiol during the estrus stage in pubescent females. In addition treatment with fluoxetine during puberty in females had a lasting effect on corticosterone levels in adulthood. During puberty both saline and fluoxetine treated males had higher

levels of corticosterone than females. In adulthood, saline treated females had an increase in corticosterone levels that was blocked by pubescent treatment with fluoxetine. This is of particular interest as at the time of sacrifice the subjects were adults had not been exposed to the drug for 29 days. These data suggest that female rats treated with fluoxetine during puberty may have an abnormal stress response in adulthood. The effects of fluoxetine on corticosterone levels were dissociated from effects on neurogenesis, as these females did not show altered levels of neurogenesis. In general this data contributes to a growing body of evidence that corticosterone and stress affect male but not female neurogenesis (Falconer and Galea, 2003; Shors et al., 2007).

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Sex differences in fluoxetine treatment: possible mechanisms

Chronic fluoxetine treatment with a dose (5mg/kg) that increased cell proliferation and neurogenesis in the adult male rat did not increase cell proliferation or neurogenesis in female rats. Longer periods of treatment (21 days) with higher doses of fluoxetine (10 mg/kg & 25 mg/kg) did increase cell proliferation in the female mouse (Engesser-Cesar et al., 2007; Lagace et al, 2007). In one study chronic treatment of 10mg/kg increased cell proliferation in both male and female mice 2hrs after BrdU injection (Lagace et al, 2007). A second study in the female mouse indicated that only very high doses of fluoxetine increased cell proliferation. Female mice treated for 21 days with 25 mg/kg of fluoxetine had increased cell proliferation 24 hrs after last BrdU injection (7 days of injections). Female mice treated with 5mg/kg or 10mg/kg did not have an increase in cell proliferation (Engesser-Cesar et al., 2007). It should be noted that the clinically relevant dose in a human is 0.15mg/kg (Wyneken et al., 2006). The half

life of fluoxetine is 12 fold lower in the mouse than the human; 4-5hrs for the mouse, 48 hrs in humans (Alvarez et al., 1998) indicating that higher doses are necessary in rodents to be comparable to the same dose in humans. Even so this would result in a clinically relevant dose of 1.8mg/kg in the rodent. The dose of 5mg/kg used in the current study was higher than the clinically relevant dose and was unable to increase neurogenesis in female rats. While the dose used in the current study did not increase neurogenesis in adult female rats it has been demonstrated to alter stress related behavior suggesting that it is an effective dose. Treatment for a minimum of 14 days with 5mg/kg of fluoxetine blocked the effects of acute stress on trace eyeblink conditioning in female rats (Leuner et al., 2004) suggesting that while this dose is not sufficient to affect neurogenesis it is sufficient to ameliorate the effects of stress on a cognitive task which requires the hippocampus.

Sex differences in the effects of fluoxetine treatment may arise from hormonal mediators of neurogenesis. In the female rats estrogen replacement after ovariectomy increased neurogenesis. Ovariectomy decreases cell proliferation but the effects may be transient. Short term (6 d) but not long term (21 d) estrogen depletion decreased cell proliferation (Banasr et al, 2001) A separate study indicated long term (28-35 d) effects of ovariectomy on neurogenesis when subjects were compared with sham operated females in proestrus but not diestrus (Tanapat et al., 2005). In ovariectomized rats, estrogen replacement increased cell proliferation within 4 hours of replacement, but reduced cell proliferation when it was measured 48 hrs after estrogen treatment (Ormerod et al., 2002a). The subsequent decrease in cell proliferation was dependent on the presence of adrenal hormones as estrogen did not increase cell proliferation in

adrenalectomized females. Long term steady level estrogen replacement does not increase cell proliferation and progesterone treatment after short-term estradiol replacement decreases cell proliferation (Tanapat et al., 2005). It is thought that short term estrogen treatment is more similar to the fluctuations across the intact cycle. The short term estrogen related increase in cell proliferation is dependent on the presence of 5HT (Banasr et al., 2001). Estrogen did not increase cell proliferation in rats that were 5HT depleted by p-chlorophenylalanine (PCPA). Furthermore, increasing 5HT by administration of 5-hydroxytrptophan (5-HTP) increased cell proliferation in ovariectomized rats in the absence of estrogen. There were no additive effects of ovariectomy and PCPA treatment which suggest that 5HT and estrogen use a common pathway in females to increase cell proliferation.

Two forms of the estrogen receptor are involved with the effects of estrogen on cell proliferation, however ER β has a predominant influence (Mazzucco et al., 2006). In ovariectomized rats all doses of an ER β agonist increased cell proliferation, whereas only the highest dose of an ER α agonist did so. Furthermore ER β mRNA was found to be co-localized more often than ERα mRNA, with Ki 67 a marker of mitotic division. It should be noted that estrogen replacement increased cell proliferation to a greater degree than treatment with either agonist suggesting other mechanisms are involved

In ovariectomized female rats chronic treatment with estrogen increased [^{3}H] paroxetine binding in the hippocampus (Kranjnak et al., 2003). Chronic high doses estradiol but not estradiol + progestesterone decreased the number of 5HT1A receptors in the dentate gyrus of the hippocampus (Birzniece et al., 2001). Furthermore, estradiol + progesterone, but not estradiol alone increased receptor density in area CA1 suggesting a

complex relationship between ovarian hormones and 5HT1A receptor density in the hippocampus. Acute treatment with estradiol did not alter 5HT1A receptor density in the hippocampus, whereas it did down-regulate receptor density in the medial amygdala (Osterlund et al., 1998). Intact adult females have higher receptor density in the hippocampus than males, but there were no sex differences in binding affinity (Stamatakis et al., 2006). Sex differences in 5HT 1A receptor density occur prior to puberty (28d) but are linked to endogenous levels of testosterone rather than estrogen (Zhang et al., 1999). Castration of male rats increased 5HT1A receptor density and application of exogenous testosterone, decreased density to the levels determined in intact males. Again regardless of differences in the receptor density there were no sex differences in binding affinity. Together these data indicate that under normal conditions, females have more 5HT1A receptors than males, but with no differences in the ability of these receptors to bind 5HT. To date, no studies have examined the relationship between 5HT1A receptors and neurogenesis in females. Given the relationship between serotonin and estrogen on cell proliferation in females (Banasr et al, 2001) it would be informative to determine if this receptor is involved in the effects of estrogen on neurogenesis.

Metabolism may also be a factor in the sex difference reported here for the effects of fluoxetine on neurogenesis. Female rats have higher levels of 5HT and its metabolite 5H1AA (Carlsson & Carlsson 1988). Measured levels of 5HT and 5H1AA levels in the hippocampus were also sexually dimorphic with females displaying higher levels of both. Synthesis of 5HT in the hippocampus was 53% greater for females compared to males (Haleem et al., 1990). Application of 8-OH-DPAT the 5HT1A agonist decreased 5HT synthesis to a greater degree in females (64%) than males (32%). Therefore female rats

are similar to prepubescent rats (PND 26) in their turnover rate of 5HT (Cabera-Vera et al., 1997) and rate of metabolism may be a factor in age and sex related differences in the effects of antidepressant treatment. However it should be noted that PET scans of healthy male and female humans measuring uptake of a form of labeled tryptophan, a precursor to 5HT which accumulates in serotonin neurons, indicated that males have 52% greater mean rate of 5HT synthesis than females (Nishizawa et al., 1997). Studies measuring 5HT synthesis in CSF for humans have indicated the opposite effects, with females displaying higher amounts of tryptophan and a higher rate of 5HT of synthesis than males (Young et al., 1980). Discrepancies among these studies may result from the metabolites of 5HT remaining longer in the CSF of females.

Depletion of 5HT induced a greater decrease in levels of 5HT in females than males (Nishizawa et al., 1997). Women currently under treatment for depression have also experience a greater number of depressive responses during tryptophan depletion than males (Moreno et al., 2006). For healthy subjects depletion of serotonin did induce a greater number of negative feeling than positive and a lowering of mood as indicated by the bipolar profile of mood states (POMS) and the visual analog mood scale (VAMS) in healthy females, but not males (Ellenbogen et al, 1996). Together these studies suggest that regardless of circulating levels of 5HT the female system has an increased response to alterations. This is further supported by the report of sex differences in response to antidepressants (Martenyi et al., 2001). Young but not older women (>44 years) had a better response to fluoxetine treatment than a tricyclic antidepressant. Males and older women did not differ in response between the treatments. In addition studies of adolescents have determined that SSRIs are the preferred route of treatment. A meta

analysis of antidepressant treatments determined that the SSRI class antidepressants did have a significant benefit over placebo, where as tricyclic antidepressants did not (Papanikalaou et al., 2006). Given that the 2 populations (females/ adolescents) that are the ones most benefited by this class of antidepressant in humans, and in animal models differ from adult males in measures of neurogenesis, metabolism, and receptor density, we need to question whether the fluoxetine induced increase in neurogenesis is an epiphenomenon and not a mechanism of drug efficacy.

Neurogenesis theory of depression?

The neurogenesis theory of depression suggests that stress related to depression results in an overall decrease in hippocampal plasticity and function (Jacobs et al., 2000; Duman, 2004a; Sapolsky, 2004). This decrease in plasticity may in turn relate to the decrease in the cognitive abilities of depressives (Vythilingham et al., 2004). It has been proposed that decreased hippocampal function would result in a decreased awareness of context and episodic memory resulting in a generalization of negative experience (Becker & Wojtowicz, 2006). The hippocampus is involved in a negative feedback loop with the HPA axis and the amygdala, so hippocampal dysfunction would result in deregulation of the biological stress responses. As around half of depressives also display hypercortisolemia this is a possible mechanism of dysfunction in those individuals (Sapolsky 2004). Therefore an increase in function in the hippocampus by antidepressant treatment would increase contextual perception and attenuate stress responses to external stimuli. Stress and depression may act on a variety of growth factors such as BDNF and these in turn would affect neurogenesis along with other measures of plasticity. These

suppositions stemmed from a series of studies in adult male rats which determined that antidepressants increase cell proliferation and neurogenesis (Encinas et al., 2006; Malberg et al., 2000), and that in stressed subjects, antidepressants reverse stress related decreases in neurogenesis (Chen et al., 2006; Malberg & Duman, 2003; but see Vollmayr et al., 2003) . Here we report that antidepressant treatment does not increase cell proliferation or neurogenesis in pubescent or adult female subjects. However we are unable to address whether treatment would reverse stress related deficits in neurogenesis. Male rats that underwent injections had higher levels of corticosterone than naïve controls, suggesting that the injection paradigm may have acted as a chronic mild stressor. Injections did not increase corticosterone levels in females. An attempt was made to affect cell proliferation through exposure to an acute stressor but it was unsuccessful as the stressor did not decrease cell proliferation.

The neurogenesis theory of depression suggests a causal relationship between decreased neurogenesis and depression. Therefore manipulations which decrease neurogenesis should induce depressive symptoms. Irradiation of the hippocampus in male mice severely reduced cell proliferation (~85% reduction) but did not induce any behavioral indications of depression (Santarelli et al, 2003). Administration of PCPA or a serotonergic toxin decreased cell proliferation in female but not male rodents (Brezun et al., 1999 ; Huang & Herbert, 2005b) therefore reducing 5HT should reduce neurogenesis at least in females. In humans administration of a tryptophan depleting drink of amino acids to reduce 5HT only induced feelings of depression in subjects undergoing drug treatment or in remission after drug treatment as measured on the Hamilton Rating Scale for Depression (HAM-D) (Heninger et al., 1996; Delgado & Moreno, 2000). Feelings of

depression were not induced by serotonin depletion in non depressives and un-medicated depressives did not experience a worsening of depressive symptoms suggesting that low levels of serotonin may not be the biological basis for depression (Heninger et al., 1996). The type of antidepressant used for treatment interacted with symptom relapse further supporting the idea that serotonin deregulation is not necessarily the cause of depression (Delgado & Moreno, 2000). The majority of subjects treated with SSRIs or MAOIs relapsed after depletion (90%), whereas those treated with norepinepherine related antidepressants had a much lower incidence of relapse (20-25%). Depletion of catecholamines using alpha- methyl-para-tyrosine (AMPT) resulted in opposite effects; patients who were treated with desipramine had a greater incidence of relapse (80%) than those treated with fluoxetine (20-25%). Therefore rather than causing depression, serotonin and neurogenesis may have a greater involvement in treating depression. As decreases in 5HT levels may not be the cause of depression, it is therefore unlikely that the resulting decrease in neurogenesis would be a mechanism in depression. This is especially the case as the group of animals (females) who have a demonstrated decrease in neurogenesis as a result of depleted 5HT (Brezun et al., 1999) do not have increased neurogenesis after antidepressant treatment. These data suggest that the neurogenesis theory of depression may not be valid.

If we are to accept the neurogenesis theory of depression than an alternative explanation for the reported data is that the increase in neurogenesis induced by fluoxetine treatment in males is not relevant for the effects of these drugs during puberty or in females. This argument stems from findings on the effects of antidepressants on learned helplessness behavior and the involvement of the norepinepherine system with

stress. The learned helplessness behavioral paradigm involves exposing animals to controllable or uncontrollable stress and then testing their abilities to actively avoid a subsequent stressor (Overmier & Seligman, 1967). The concept behind the paradigm was that animals learned that they had no control over the situation and therefore developed a motivational deficit and ceased to actively respond. The inescapable stress used in learned helplessness depletes norepinepherine and impairs active avoidance responding (Weiss et al., 1970). However animals exposed to controllable stress of the same stressor intensity and duration have less depletion of NE and increased active avoidance. Antidepressant treatment of various types increases active avoidance in the LH paradigm for adult male rats (Zazpe et al., 2006). Furthermore SSRI antidepressants reverse the stress induced decreases in neurogenesis reported with this paradigm (Chen et al, 2006; Malberg & Duman, 2003; but see Vollmayr et al., 2003). Therefore a relationship between neurogenesis, norepinepherine and active avoidance is likely. However females do not have the same stress related behavioral deficits in learned helplessness (Jenkins et al., 2001;Kirk & Blampied, 1985; Steenbergen et al., 1990; Shors et al., 2007). Nor do females have a chronic stress related decrease in neurogenesis (Shors et al., 2007). Therefore alterations in the NE system from stress may not have the same debilitating behavioral and physical effects in females and neurogenesis based alterations may not be necessary, as there is nothing to compensate for.

Biologically there is some basis for this explanation as sex and age differences are found in the morphology and stress response of the locus coeruleus (LC) and noradrenergic function. Stressful experience triggers the firing of noradrenergic neurons in the LC. In turn activation of these neurons results in the increased release of the

extracellular concentration of norepinepherine (NE) in the hippocampus which reduces the activation of hippocampal neurons (for review see Mongeau et al., 1997). Activation of 5HT1A receptors decrease the firing rates of LC NE neurons, so it is thought that SSRI class antidepressants increase serotonin levels and decrease NE levels, decreasing the LC's response to stress. The LC is sexually dimorphic and becomes so at puberty (Pinos et al., 2001). LC volume increases similarly for both sexes between E16 and PND 15. At PND 15 volume stabilizes for females but continues to increase for males until PND 60 when it then decreases until PND 90. In addition there is a sex differences in the number of neurons. The number of neurons increases from E16 to PND 15, from PND 15-35 a sexually dimorphic decrease occurs in the number of neurons with males displaying a larger decrease than females. From PND 35-45 a second increase occurs in the number of neurons, which continues up to PND 60 in females but stops at PND 45 in males. In adulthood females have a larger LC than males, with more neurons and display increased neuronal activity under some forms of stress (Curtis et al., 2006). In anesthetized rats hypotensive stress induced by an infusion of sodium nitroprusside for 15 min resulted in a larger increase in the discharge by LC neurons by females than males regardless of hormonal manipulations. Females were also more sensitive to application of exogenous CRF and showed a greater neuronal activation at lower doses than males. Analysis of western blots indicated that females have more CRF receptors than males. However swim stress had opposite effects in males and females. Exposure to swim stress 24 hrs prior to a hypotensive stress exposure eliminated sex differences by increasing the LC discharge in males but decreasing it in ovariectomized females treated with estrogen. These effects occurred even though baseline recordings were similar to unstressed

controls after swim stress but prior to the hypotensive challenge. This may explain why females do not show learned helplessness, in chronic stress situations. First females may have greater stores of NE as they have more neurons, second repeated stress may decrease the release of NE in females while increasing it in males.

While no study has investigated learned helplessness during puberty, it is likely that age based alterations in the norepinepherine system may also interact. The NE responses to stress changes with development in the hypothalamus (Choi & Kellogg, 1996). Restraint stress increased NE release in adults but did not alter release during puberty compared to unstressed controls, and prior to puberty restraint stress decreased NE release. All ages displayed similar corticosterone responses to stress. Pubescent males also have no neuronal response to norepinepherine re-uptake inhibition (Choi et al., 1997). Blocking re-uptake with nisoxetine did not alter NE release in the hypothalamus regardless of dose. High doses of nisoxetine decreased NE release in adult males, whereas low doses increased release. In pre-pubescent subjects low doses did not alter NE release whereas high doses increased it. These data may in part explain why NE based antidepressants are not effective during puberty (Papanikalaou et al., 2006). Together these studies strongly suggest that NE activation is different during puberty than prior to and after puberty. Therefore drugs that alter NE release will have different effects on pubescent subjects than adults. As the relationship between NE, fluoxetine and neurogenesis is unclear further research is necessary to determine whether the age differences in the effects of fluoxetine on neurogenesis involve NE.

Regardless of whether the neurogenesis theory of depression is valid, the current data determine that sex and age differences exist in the action of fluoxetine. This is a

starting point from which further experimentation can determine the mechanisms by which these responses differ and the importance of those differences for treatment efficacy.

Conclusion

The current study indicates that the same dose of fluoxetine (5mg/kg) affects pubescent and female rats differently than adult male rats: treatment increased cell proliferation and neurogenesis in adult males but not in females or either sex during puberty. Furthermore fluoxetine treatment during puberty decreased basal corticosterone levels in females when they were sacrificed after a 29 day wash out period in adulthood, indicating that there are sex specific developmental effects of fluoxetine treatment on hormones. While these data do not address whether fluoxetine treatment can increase negative emotional experiences such as suicidal thoughts in human adolescents, they do suggest that pubescent brain does not respond to treatment on a cellular level in the same way as the adult male brain. Fluoxetine is the only antidepressant currently approved for use in adolescents (FDA statement, 2004). Out of all of the current SSRI class antidepressants fluoxetine has the best risk/benefit profile, in that it has the largest effects in relieving depressive symptoms and the least risk of triggering aversive events in adolescents (Whittington et al., 2004). Therefore the drug is clearly effective in treating adolescent depression. The current data suggest that increased neurogenesis does not seem to be necessary for the efficacy of this drug during puberty.

Age altered the cellular response to antidepressant treatment; in addition sex also determined whether fluoxetine treatment increased neurogenesis. Adult male rats treated

with fluoxetine had increased neurogenesis but female rats treated with fluoxetine did not have increased cell proliferation or neurogenesis. The effects of fluoxetine treatment did not interact with the estrous cycle to increase cell proliferation in pubescent females or adult females. In humans females are twice as likely as males to suffer a depressive episode (Earls, 1987; Kessler, 2003). Prior to menopause women are more responsive to SSRI antidepressants than to tricyclic antidepressants (Martenyi et al., 2001). Depletion of serotonin has larger effects in women than men (Nishizawa et al., 1997) and causes a greater number of depressive symptoms (Moreno et al., 2006). Together these studies suggest that the female system is affected more by alterations in serotonin content than males. Given that, it is interesting that neurogenesis is not increased by fluoxetine treatment in female rats. Together these data suggest that different underlying mechanisms may be responsible for the efficacy of fluoxetine at different ages and for different sexes.

BIBLIOGRAPY

1.Abrous, D.N., Koehl, M., Moal, M.L.(2005) Adult Neurogenesis: From precursors to network and physiology. Physiology Reviews, 85, 523-69.

2. Alvarez, J.C., Dothua, D., Collignon, I., Adernier, C., Spreux-Varoquaux, O. (1998) Determination of fluoxetine and its metabolite norfluoxetine in serum and brain areas using high-performance liquid chromatography with ultraviolet detection. Journal of Chromatography B, 707, 175-80.

3. Ansorge, M.S., Zhou, M., Lira,A., Hen, R., Gingrich, J.A. (2004) Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice. Science, 306, 879-81.

4. Atkinson, H.C., Waddell, B.J. (1997) Circadian variation in basal plasma corticosterone and adrenocorticotropin in the rat: Sexual dimorphism and changes across the estrous cycle. Endocrinology, 138, 9, 3842-48.

5. Banasr, M., Hery, M., Brezun, J.M., Daszuta, A. (2001) Serotonin mediates oestrogen stimulation of cell proliferation in the adult dentate gyrus. European Journal of Neuroscience, 14, 1417-24.

6. Becker, S., Wojtowicz, J.M. (2007) A model of hippocampal neurogenesis in memory and mood disorders. Trends in Cognitive Science, 11, 2, 70-6.

7. Birzniece, V., Johansson, I.M., Wang, M.D., Secki, J.R., Backstrom, T., Olsson, T. (2001) Serotonin 5-HT $_{1A}$ receptor mRNA expression in the dorsal hippocampus and raphe nuclei after gonadal hormone manipulation in female rats. Neuroendocrinology, 74, 135-42.

8. Blier, P., de Montigny, C., Chaput, Y. (1987) Modifications of the serotonin system by antidepressant treatments: implications for the therapeutic response in major depression. Journal of Clinical Psychopharmacology, 7, 6 supplemental, 24S-35S.

9. Brezun, J. M. and Daszuta, A. (1999). Depletion in serotonin decreases neurogenesis in the dentate gyrus and subventricular zone of adult rats. Neuroscience 89, 999-1002.

10. Cabrera-Vera, T.M., Garcia, F., Pinto, W., Battaglia, G. (1997) Effect of prenatal fluoxetine (Prozac) exposure on brain serotonin neurons in prepubescent and adult male rat offspring. The Journal of Pharmacology and Experimental Therapeutics, 280, 1, 138-45.

11. Cameron, H. A. and Gould, E. (1994). Adult neurogenesis is regulated by adrenal steriods in the dentate gyrus. Neuroscience 61, 203-209.

12. Cameron, H. A., Tanapat, P., and Gould, E. (1998). Adrenal steriods and N-Methyl-D-Aspartate receptor activation regulate neurogenesis in the dentate gyrus of adult rats through a common pathway. Neuroscience 82, 349-354.

13. Cameron, H. A. and McKay, R. D. G. (2001). Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. The Journal of Comparative Neurology 435, 406-417.

14. Carlsson, M., Carlsson, A. (1988) A regional study of sex differences in rat brain serotonin. Progress in Neuro-Psychopharmacology and Biological Psychiatry, 12, 1, 53-61.

15. Chalmers, D.T., Kwak, S.P., Mansour, A., Akil, H., Watson, S.J. (1993) Corticosteroids regulate brain hippocampal $5HT_{1A}$ receptor mRNA expression. The Journal of Neuroscience, 1, 3, 914-23.

16. Chen, H., Pandey, G.N., Dwivedi, Y. (2006) Hippocampal cell proliferation regulation by repeated stress and antidepressants. Neuroreport, 17, 9, 863-67.

17. Choi, S., Kellogg, C.K. (1996) Adolescent development influences functional responsiveness of noradrenergic projections to the hypothalamus in male rats. Developmental Brain Research, 94, 144-51.

18. Choi, S., Kellogg, C.K. (1997) Control of endogenous norepinepherine release in the hypothalamus of male rats changes over adolescent development. Developmental Brain Research, 98, 134-41.

19. Christie, B.R., Cameron, H.A. (2006) Neurogenesis in the adult hippocampus. Hippocampus, 16, 199-207.

20. Cooke, B.M., Woolley, C.S. (2005) Sexually dimorphic synaptic organization of the medial amygdala. The Journal of Neuroscience, 25, 46, 10759-67.

21. Couzin, J. (2004). Volatile chemistry: Children and antidepressants. Science 305, 468-470.

22. Curtis, A.L., Bethea, T., Valentino, R.J. (2006) Sexually dimorphic responses of the brain norepinepherine system to stress and corticotropin – releasing factor, Neuropsychopharmacology, 31, 544-54.

23. Czeh, B., Lucassen, J. (2007) What causes the hippocampal volume decrease in depression? Are neurogenesis, glial changes and apoptosis implicated. European Archives of Psychiatry and Clinical Neuroscience, Epub ahead of print, April 1.

24. Dayer, A.G., Ford, A.A., Cleaver, K.M., Yassaee, M., Cameron, H.A. (2003) Short-term and long-term survival of new neurons in the rat dentate gyrus. The Journal of Comparative Neurology, 460, 563-72.

25. De Vry, J. (1995) 5-HT1A receptor agonists: recent developments and controversial issues. Psychopharmacology, 121,1, 1-26.

26. Delgado, P.L., Moreno, F.A. (2000) Role of norepinepherine in depression. Journal of Clinical Psychiatry Supplemental, 1, 5-12.

27.Dewar, K.M., Grondin, L., Nenonene, E.K., Ohayon, M., Reader, T.A. (1993) [3H]paroxetine binding and serotonin content of rat brain: absence of changes following antidepressant treatments. European Journal of Pharmacology, 235,1,137-42.

28. Drevets, W.C., Frank, E, Price, J.C., Kupfer, D.J., Holt, D., Greer, P.J., Huang, Y., Gautier, C., Mathis, C.(1999) PET imaging of serotonin 1A receptor binding in depression. Biological Psychiatry,46,10, 1375-87.

29. Duman, R.S. (2004a) Depression: A case of neuronal life and death? Biological Psychiatry, 56, 3, 140-145.

30. Duman, R.S. (2004b) Role of neurotrophic factors in the etiology and treatment of mood disorders. Neuromolecular Medicine, 5,1, 11-25.

31. El Masry, S.E.D., Cohen, G.M., Mannering, G.J. (1974) Sex-dependent differences in drug metabolism in the rat. The American Society for Pharmacology and Experimental Therapeutics, 2,3, 267-78.

32. Ellenbogen, M.A., Young, S.N., Dean, P., Plamour, R.M., Benkelfat, C. (1996) Mood response to acute tryptophan depletion in healthy volunteers: Sex differences and temporal stability. Neuropsychopharmacology, 15,5, 465-74.

33. Encinas, J.M., Vaahtokari, A., Enikolopov, G. (2006) Fluoxetine targets early progenitor cells in the adult brain. Proceedings of the National Academy of Sciences, 103, 21, 8233-38.

34. Engesser-Cesar, C., Anderson, A.J., Cotman,C.W. (2007) Wheel running and fluoxetine antidepressant treatment have differential effects in the hippocampus and the spinal cord. Neuroscience, 144, 1033-44.

35. Earls, F., 1987. Sex differences in psychiatric disorders: origins and developmental influences. Psychiatry Developments, 5, 1-23.

36. Falconer, E.M., Galea, L.A.M. (2003) Sex differences in cell proliferation, cell death and defensive behavior following acute predator odor stress in adult rats. Brain Research, 975, 22-36.

37. FDA statement. FDA statement on recommendations of the psychopharmacologic drugs and pediatric advisory commitees. 2004.

38. Galea, L.A., Spritzer, M.D., Barker, J.M., Pawluski, J.L. (2006) Gonadal hormone modulation of hippocampal neurogenesis in the adult. Hippocampus, 16, 225-32.

39. Geidd, J. N., Castellanos, F. X., Rajapakse, J. C., Vaituzis, A. C., and Raporort, J. L. (1997). Sexual dimorphism if the developing human brain. Prog. Neuro-Psychopharmacol. & Biol. Psychiat. 21, 1185-1201.

40. Giedd, J. N., Vaituzis, A. C., Hamburger, S. D., Lange, N., Rajapakse, J. C., Kaysen, D., Yauss, Y. C., and Rapoport, J. L. (1996). Quantitative MRI of the temporal lobe, Amygdala, and Hippocampus in normal human development: Ages 4-18 years. The Journal of Comparative Neurology 366, 223-230.

41. Gogtay, N., Giedd, J. N., Lusk, L., Hayashi, K. M., Greenstien, D., Vaituzis, A. C., Nugent III, T. F., Herman, D. H., Clasen, L. S., Toga, A. W., Rapoport, J. L., and Thompson, P. M. (2004). Dynamic mapping of human cortical development during early childhood through early adulthood. Proc Natl Acad Sci USA. 101, 8174-8179.

42. Gould, E., Cameron, H. A., Daniels, D. C., Woolley, C. S., and McEwen, B. S. (1992). Adrenal hormones supress cell division in the adult rat dentate gyrus. The journal of neuroscience 12, 3642-3650.

43. Gould,E., Beylin, A., Tanapat, P., Reeves, A., Shors, T.J. (1999) Learning enhances adult neurogenesis in the hippocampal formation. Nature Neuroscience, 2, 260-5.

44. Hajszan, T. and Leranth, C. (2005). Short-term treatment with the antidepressant fluoxetine triggers pyramidal dendritic spine synapse formation in rat hippocampus. European Journal of Neuroscience 21, 1299-1303.

45. Haleem, D.J., Kennett,G.A., Curzon, G. (1990) Hippocampal 5-hydroxytryptamine synthesis is greater in female rats than in males and more decreased by the 5-HT1A agonist 8-OH-DPAT. Journal of Neural Transmission, General Section, 79, 1-2, 93-101.

46.Hervas, I., Queiroz, C.M., Adell, A., Artigas, F.(2000) Role of uptake inhibition and autoreceptor activation in the control of 5-HT release in the frontal cortex and dorsal hippocampus of the rat. British Journal of Pharmacology, 130,1,160-6

47. Hayes, N.L., Nowakowski,R.S.(2002) Dynamics of cell proliferation in the adult dentate gyrus of two inbred strains of mice. Developmental Brain Research, 134, 77-85.

48. Heine, V. M., Maslam, S., Joels, M., and Lucassen, P. J. (2004). Prominent decline of newborn cell proliferation, differentiation, and apoptosis in the aging dentate gyrus, in absence of age-related hypothalamus-pituitary-adrenal axis activation. Neurobiol. Aging 25, 361-375.

49. Heninger, G.R., Delgado, P.L., Charney, D.S.(1996) The revised monoamine theory of depression: A modulatory role for monoamines, based on new findings from monoamine depletion experiments in humans. Pharmacopsychiatry, 29,1, 2-11.

50. Hervas, I., Queiroz, C.M.T., Adell, A., Artigas, F. (2000) Role of uptake inhibition and autoreceptor activation in the control of 5-HT release in the frontal cortex and dorsal hippocampus of the rat. British Journal of Pharmacology, 130, 160-66.

51. Hjorth, S., Auerbach, S.B. (1994) Lack of 5-HT1A receptor desensitization following chronic citalopram treatment, as determined by in vivo microdialysis. Neuropharmacology, 33, 3-4, 331-4.

52. Hjorth, S., Auerbach, S.B (1996) 5-HT_{1A} autoreceptors and the mode of action of selective serotonin reuptake inhibitors (SSRI). Behavioural Brain Research, 73, 281-83.
53. Hjorth, S., Bengtsson, H.J., Kullberg, A., Carlzon, D., Peilot, H., Auerbach, S.B. (2000) Serotonin autoreceptor function and antidepressant drug action. Journal of Psychopharmacology, 14,2, 177-85.

54. Hodes G.E. and Shors T.J. (2005). Distinctive stress effects on learning during puberty. Hormones and Behavior 48, 163-171.

55. Hoshaw, B.A, Connellan, J., Lucki, I. (2006) Citalopram but not desipramine produces antidepressant- like effects in adolescent rats. Abstract : Society for Neuroscience.

56. Hoyer, D., Hannon, J.P., Martin, G.R. (2002) Molecular, pharmacological and functional diversity of 5-HT receptors. Pharmacology, Biochemistry and Behavior, 71,4,533-54.

57. Huang, G.J., Herbert, J. (2006) Stimulation of neurogenesis in the hippocampus of the adult rat by fluoxetine treatment requires rhythmic changes in corticosterone. Biological Psychiatry, 59,7, 619-24.

58. Huang, G.J., Herbert, J.(2005a) Serotonin modulates the suppressive effects of corticosterone on proliferating progenitor cells in the dentate gyrus of the hippocampus of the adult rat. Neuropsychopharmacology, 30, 231-41.

59. Huang, G.J., Herbert, J. (2005b) The role of 5-HT_{1A} receptors in the proliferation and survival of progenitor cells in the dentate gyrus of the adult hippocampus and their regulation by corticoids. Neuroscience, 135, 803-13.

60.Jacobs, B.L., Van Praag, H., and Gage, F. H. (2000). Adult brain neurogenesis and psychiatry: a novel theory of depression. Molecular Psychiatry 5, 262-269
.
61. Jacobs, B.L. (2002) Adult brain neurogenesis and depression. Brain, Behavior, and Immunity, 16, 602-609.

62.Jenkins, J. A., Williams, P., Kramer, G. L., Davis, L. L., and Petty, F. (2001). The influence of gender and estrous cycle on learned helplessness in the rat. Biological Psychology 58, 147-158.

63. Jick, H., Kaye, J. A., and Jick, S. S. (2004). Antidepressants and the risk of suicidal behaviors. Journal of the American Medical Association 292, 338-343
.
64. Kaltiala-Henio, R., Marttunen, M., Rantanen, R., and Rimpela, M. (2003). Early puberty is associated with mental health problems in middle adolescence. Social Science and Medicine 57, 1055-1064.

65. Kessler, R. C., Avenevoli, S., and Merikangas, K. R. (2001). Mood disorders in children and adolescents: an epidemiologic perspective. Biological Psychiatry 49, 1002-1014.

66. Kessler, RC. (2003) Epidemiology of women and depression. Journal of Affective Disorder 74(1),5-13.

67. Kirk, R. C. and Blampied, N. M. (1985). Activity during inescapable shock and subsequent escape avoidance learning: Female and male rats compared. New Zeland Journal of psychology 14, 9-14.

68. Klancnik, J.M., Baimbridge, K.G., Phillips, A.G.(1989) Increased population spike amplitude in the dentate gyrus following systemic administration of 5-hydroxytryptophan or 8-hydroxy-2-(di-n-propylamino)tetralin. Brain Research, 505,1, 145-48.

69. Klimek, V., Zak- Knapik, J., Mackowiak, M.(1994) Effects of repeated treatment with fluoxetine and citalopram, 5-HT uptake inhibitors, on 5-HT_{1A} and 5-HT_2 receptors in the rat brain. Journal of Psychiatry and Neuroscience, 19,1, 63-67

70. Kornack, D.R., Rakic, P.(1999) Continuation of neurogenesis in the hippocampus of the adult macaque monkey. Proceedings of the National Academy of Science USA, 96,10, 5768-73..

71. Kranjnak, K., Rosewell, K.L., Duncan, M.J., Wise, P.M. (2003) Aging, estradiol and time of day differently affect serotonin transporter binding in the central nervous system of female rats. Brain Research, 990, 87-94.

72. Kuehn, B.(2007) FDA panel seeks to balance risks in warnings for antidepressants. Journal of the American Medical Association, 297,6, 573-74.

73. Lagace, D.C., Fischer, S.J., Eisch, A.J.(2007) Gender and endogenous levels of estradiol do not influence adult hippocampal neurogenesis in mice. Hippocampus, 17, 175-80.

74. Landry, M., Frasier, M., Chen, Z., Van de Kar, LD., Zhang, Y., Garcia, F., Battaglia, G.(2005) Fluoxetine treatment of prepubescent rats produces a selective functional reduction in the 5-HT2A receptor-mediated stimulation of oxytocin. Synapse, 58,2, 102-9.

75. Larsson, L.G., Renyi, L., Ross, S.B., Svensson, B., Angeby-Moller, K.(1990) Different effects on the response of functional pre and post synaptic 5-HT1A receptors by repeated treatment of rats with the 5-HT1A receptor agonist 8-OH-DPAT. Neuropharmacology, 29,2,86-91
.
76. Lee, H.J., Yim, S.V., Kim, M.J., Kim, Y.J., Kim, C.J., Chung, J.H.(2001) Fluoxetine enhances cell proliferation and prevents apoptosis in dentate gyrus of maternally separated rats. Molecular Psychiatry, 6,725-8.

77. Lesch, K.P. (2001) Serotonergic gene expression and depression: implications for developing novel antidepressants. Journal of Affective disorders 62, 57-76.

78. Leuner, B., Mendolia-Loffredo, S., Shors, T.J.(2004) Males and females respond differently to controllability and antidepressant treatment. Biological Psychiatry, 56,12,964-70.

79. Lupien, S.J., Evans, A., Lord,C., Miles, J., Pruessner, M., Pike, B., Pruessner, J.C. (2007) Hippocampal volume is a variable in young as in older adults: Implications for the notion of hippocampal atrophy in humans. NeuroImage, 34, 479-85.

80.Malberg, J. E., Eisch, A. J., Nestler, E. J., and Duman, R. S. (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. The journal of neuroscience 20, 9104-9110.

81. Malberg, J. E. and Duman, R. S. (2003). Cell proliferation in adult hippocampus is decreased by inescapable stress: reversal by fluoxetine treatment. Neuropsychopharmacology 28, 1562-1571.

82. Marcotte, D., Fortin, L., Potivn, P., and Papillon, M. (2002). Gender differences in depressive symptoms during adolescence: role of gender-typed characteristics, self-esteem, body image, stressful life events and pubertal status. Journal of emotional and behavioral disorders 10, 29-43.

83. Martenyi, F., Dossenbach, M., Mraz, K, Metcalfe, S. (2001) Gender differences in the efficacy of fluoxetine and maprotiline in depressed patients: a double blind trial of antidepressants with sertonergic or norepinephrinergic reuptake inhibition profile. European Neuropsychopharmacology, 11, 227-32.

84. Maswood, S., Truitt, W., Hotema, M., Caldarola-Pastuszka, Uphouse, L. (1999) Estrous cycle modulation of extracellular serotonin in mediobasal hypothalamus: role of the serotonin transporter and terminal autorecptors. Brain Research, 831, 146-154.

85. Matuszczyk, J.V., Larsson, K., Ericksson, E.(1998) Subchronic administration of fluoxetine impairs estrous behavior in intact female rats. Neuropsychopharmacology, 19,6, 45-54.

86. Mazzucco, C.A., Lieblich, S.E., Bingham, B.I., Williamson, M.A., Viau, V., Galea, L.A.M. (2006) Both estrogen receptor α and estrogen receptor β agonists enhance cell proliferation in the dentate gyrus of adult female rats. Neuroscience, 141, 1793-1800.

87. McDonald, H. Y. and Wojtowicz, J. M. (2005). Dynamics of neurogenesis in the dentate gyrus of adult rats. Neuroscience Letters 385, 70-75.

88. Mendelson, S.D., McEwen, B.S. (1991) Autoradiographic analyses of the effects of restraint-induced stress on 5-HT_{1A} , 5-HT_{1C} and 5-HT_2 receptors in the dorsal hippocampus of male and female rats. Neuroendocrinology, 54, 454-61.

89. Mendelson, S.D., McEwen, B.S. (1992) Autoradiographic analyses of the effects of adrenalectomy and corticosterone on 5-HT1A and 5-HT1B receptors in the dorsal hippocampus and cortex of the rat. Neuroendocrinology, 55, 4, 444-50.

90. Miller, M.W., Nowakowski, R.S. (1988) Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. Brain Research, 457, 1, 44-52.

91. Moll, G.H., Mehnert, C., Wicker, M., Bock, N., Rothenberger, A., Ruthger, E., Huether, G. (2000)Age-associated chances in the densities of presynaptic monamine transporters in different regions of the rat brain from early juvenile life to late adulthood. Developmental Brain Research, 119, 251-257.

92. Monroy, J., Ayala, M.E., Chavira, R., Damian-Matsumura, P., Dominguez, R. (2003) Comparative effects of injection 5,6-dihydroxtryptamine in the dorsal or medial raphe nuclei on rat puberty. Brain Research Bulletin, 60, 307-315.

93. Mongeau, R., Blier,P., de Montigny, C.(1997) The serotonergic and noradrenergic systems of the hippocampus: Their interactions and the effects of antidepressant treatments. Brain Research Reviews, 23, 145-95.

94. Morelli, E., Ansorge, M.S., Gingrich, J.A. (2006) The effect of developmental exposure to fluoxetine, citalopram, clomipramine, and desipramine on adult emotional behavior in mice. Abstract: Society for Neuroscience.

95. Moreno, F.A., McGahuey, C.A., Freeman, M.P., Delgado, P.L. (2006) Sex differences in depressive response during monoamine depletions in remitted depressive subjects. Journal of Clinical Psychiatry, 67, 1618-23.

96. Nestler, E.J., Barrot, M., DiLeone,R.J., Eisch, A.J., Gold, S.J., Monteggia, L.M. (2002) Neurobiology of depression. Neuron, 34, 13-25.

97. Nishizawa, S., Benkelfat,C., Young, S.N., Leyton, M., Mzengeza, S., de Montigny, C., Blier, P., Diksc, M. (1997) Differences between males and females in rates of

serotonin synthesis in human brain. Proceedings of the National Academy of Sciences, USA, 94, 5308-13.

98. Norrholm, S. D. and Ouimet, C. C. (2000). Chronic fluoxetine administration to juvenile rats prevents age associated dendritic spine proliferation in hippocampus, Brain Research, 883, 205-215.

99.Nowakowski, R.S., Lewin, S.B., Miller M.W.(1989) Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. Journal of Neurocytology,3, 311-8.

100. Ojeda, S. R. and Urbanski, H. F. (1994). Puberty in the rat. In E. N. J. D. Knobil (Ed.), The Physiology of Reproduction, pp. 363-409. Raven Press, New York.

101. Ormerod, B.K., Lee, T.T-Y., Galea, L.A.M. (2003a) Estradiol initially enhances but subsequently suppresses (via adrenal steroids) granule cell proliferation in the dentate gyrus of adult female rats. Journal of Neurobiology, 55,2, 247-60.

102. Ormerod, B.K., Galea, L.A.(2003b) Reproductive status influences the survival of new cells in the dentate gyrus of adult male meadow voles. Neuroscience Letters, 346, 1-2, 25-8.

103. Osterlund, M.K., Hurd, Y.L. (1998) Acute 17β- estradiol treatment down-regulates serotonin $5HT_{1A}$ receptors mRNA expression in the limbic system of female rats. Molecular Brain Research, 55, 169-72.

104. Overmier, J.B., Seligman, M.E.P. (1967) Effects of inescapable shock upon subsequent escape and avoidance responding. Journal of Comparative and Physiological Psychology, 63,1, 28-33.

105. Papanikolaou, K., Richardson, C., Pehlivanidis, A., Papadopoulou-Daifoti, Z. (2006) Efficacy of antidepressants in child and adolescent depression: A meta-analytic study. Journal of Neural Transmission, 113,3, 399-415.

106. Paus, T., Zijdenbos, A., Worsley, K., Collins, D. L., Blumenthal, J., Giedd, J. N., Rapoport, J. L., and Evans, A. C. (1999). Structural maturation of neural pathways in children and adolescents: in vivo study. Science 283, 1908-1911.

107. Perfilieva, E., Risedal, A., Nyberg, J., Johansson, B.B., Eriksson, P.S.(2001) Gender and strain influence on neurogenesis in dentate gyrus of young rats. Journal of Cerebral Blood Flow and Metabolism, 21, 211-17.

108. Piccinelli, M. and Wilkenson, G. (2000). Gender differences in depression. British Journal of Psychiatry 177, 486-492.

109. Pinos, H., Collado, P., Rodriguez-Zafra, M., Rodriguez, C., Segovia, S., Guillamon, A. (2001) The development of sex differences in the locus coeruleus of the rat. Brain Research Bulletin, 56,1,73-78.

110. Rao, M.S., Shetty, A.K.(2004) Efficacy of doublecortin as a marker to analyze the absolute number and dendritic growth of newly generated neurons in the adult dentate gyrus. European Journal of Neuroscience, 19, 234-46.

111. Rasika, S., Nottebohm, F., Alvarez-Buylla, A. (1994) Testosterone increases the recruitment and/or survival of new high vocal center neurons in adult female canaries. Proceedings of the National Academy of Sciences, USA, 91,17, 854-8.

112. Reif, A., Fritzen, S., Finger, M., Strobel, A., Lauer, M., Schmitt, A., Lesch, K.P.(2006) Neural stem cell proliferation is decreased in schizophrenia, but not depression. Molecular Psychiatry, 1-9.

113. Ressler, K.J., Nemeroff, C.B. (2000) Role of serotonergic and noradrenergic systems in the pathophysiology of depression and anxiety disorders. Depression and Anxiety, 12, Supplement 1, 2-19.

114. Romeo RD, Lee SJ, Chhua N, McPherson CR, and McEwen BS (2004). Testosterone cannot activate an adult-like stress response in prepubertal male rats. Neuroendocrinology. 79, 125-132.

115. Romeo, R. D., Lee, S. J., and McEwen, B. S. (2005). Differential stress reactivity in intact and ovariectomized prepubertal and adult female rats. Neuroendocrinology. 80, 387-393.

116. Rosenbrock, H., Bloching, A., Weiss, C., and Borsini, F. (2005). Partial serontonergic denervation decreases pregenitor cell proliferation in the adult rat hippocampus, but has no effect on rat behavior in the forced swimming test. Pharmacology, Biochemisty, and Behavior 80, 549-556.

117. Ruiz, A., Blanco, R., Santander ,J., Miranda, E.,2000. Relationship between sex differences in onset of schizophrenia and puberty. Journal of Psychiatry Research, 34,349-53.

118. Rygula, R., Abumaria, N., Domenici, E., Hiemke, C., Fuchs, E.(2006) Effects of fluoxetine on behavioral deficits evoked by chronic social stress in rats. Behavioral Brain Research, 174,1,188-92.

119. Sairanen M, Lucas G, Ernfors P, Castren M, and Castren E (2005). Brain-derived neurotrophic factor and antidepressant drugs have different but coordinated effects on neuronal turnover, proliferation, and survival in the adult dentate gyrus. J Neurosci. 25, 1089-1094.

120. Santerlli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., Weisstaub, N., Lee, J., Duman, R. S., Arancio, O., Belzung, C., and Hen, R. (2003). Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301, 805-809.

121. Sapolsky, R.M., Krey, L.C., McEwen, B.S. (1984) Glucocorticoid-sensitive hippocampal neurons are involved in terminating the adrenalcortical response. Proceedings of the National Academy of Sciences, USA, 81, 6174-77.

122.Sapolsky RM (2004). Is impaired neurogenesis relevant to the affective symptoms of depression? Biol Psychiatry 56, 137-139.

123. Serra, M., Giuseppina Pisu, M., Muggironi, M., Pardo, V., Papi, G., Sari, R., Dazzi, L., Spiga, F., Purdy, R.H., Biggio, G. (2001) Opposite effects of short versus long term administration of fluoxetine on the concentrations of neuroactive steroids in rat plasma and brain. Psychopharmacology, 158, 48-54.

124. Shapiro, B.H., Agrawal, A.K., Pampori, N.A.(1995) Gender differences in drug metabolism regulated by growth hormone. International Journal of Biochemistry and Cell Biology, 27,1, 9-20.

125. Sheline, Y.I., Wang, P.O., Gado, M.H. Csernansky, J.G., Vannier, M.W. (1996) Hippocampal atrophy in recurrent major depression. Proc. Natl. Acad. Sci.USA. 93, 3908-3913.

126. Shors, T.J., Mathew, J., Sisti, H.M., Edgecomb, C., Beckoff, S., Dalla, C. (2007) Neurogenesis and helplessness are mediated by controllability in males but not females. Biological Psychiatry, Epub. Feb 15.

127. Sisk, C.L., Zehr, J.L. (2005) Pubertal hormones organize the adolescent brain and behavior. Frontiers in Neuroendocrinology, 26,3-4,163-74.

128. Sonnenberg, C.M., Beekman, A.T.F, Deeg, D.J.H., Van Tilburg, W., 2000. Sex differences in late-life depression. Acta Psychiatry Scandinavia, 101, 286-92.

129.Stamatakis, A., Mantelas, A, Papaioannou, A., Pondiki, S., Fameli, M., Stylianopoulou, F. (2006) Effect of neonatal handling on serotonin 1A sub-type receptors in the rat hippocampus. Neuroscience, 140, 1-11.

130. Stockmeier, C.A., Mahajan, G.J., Konick, L.C., Overholser, J.C., Jurjus, G.J., Meltzer, H.Y., Uylings, B.M., Friedman, L., Rajkowska, G. (2004) Cellular changes in the postmortem hippocampus in major depression. Biol. Psych, 56, 640-650.

131. Stout, S.C., Owens, M.J., Nemeroff, C.B. (2002) Regulation of corticotropin-releasing factor neuronal systems and hypothalamic-pituitary-adrenal axis activity by

stress and chronic antidepressant treatment. Journal of Pharmacology and Experimental Therapeutics, 300,3, 1085-92.

132. Steenbergen, H. L., Heinsbroek, R. P. W., VanHest, A., and Van de Poll, N. E. (1990). Sex-dependent effects of inescapble shock administration on shuttle box escape perfomance and elevated plus maze behavior. Physiology and Behavior 48, 571-576.

133. Subhash, M.N., Srinivas, B.N., Vinod, K.Y., Jagadeesh, S.(2000) Modulation of 5HT1A receptor mediated response by fluoxetine in rat brain. Journal of Neural Transmission, 107, 3,377-87.

134. Tanapat P, Hastings NB, Reeves AJ, and Gould E. (1999). Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. J Neurosci. 19, 5792-5801.

135. Tanapat P, Hastings NB, Rydel TA, Galea LA, and Gould E (2001). Exposure to fox odor inhibits cell proliferation in the hippocampus of adult rats via an adrenal hormone-dependent mechanism. J Comp Neurol. 437, 496-504.

136. Tanapat P, Hastings NB, and Gould E (2005). Ovarian steroids influence cell proliferation in the dentate gyrus of the adult female rat in a dose- and time-dependent manner. J Comp Neurol. 481, 252-265.

137. Taupin, P.(2007) BrdU immunohistochemistry for studying adult neurogenesis: Paradigms, pitfalls, limitations and validation. Brain Research Reviews, 53,1,198-214.

138. Taylor, G., Bardgett, M., Csernansky, J., Early, T., Haller, J., Scherrer, J., Womack, S. (1996) Male reproductive systems under chronic fluoxetine or trimipramine treatment. Physiology and Behavior, 59,3, 479-485.

139. Taylor, G.T., Farr, S., Klinga, K., Weiss, J.(2004) Chronic fluoxetine suppresses circulating estrogen and the enhanced spatial learning of estrogen-treated ovariectomized rats. Psychoneuroendocrinology, 29, 1241-49.

140. Uphouse, L., Hensler, J.G., Sarkar, J., Grossie, B. (2005) Fluoxetine disrupts food intake and estrous cyclicity in Fischer female rats. Brain Research, 1072,1,79-90.

141. Van de Kar, L.D., Raap, D.K., Battaglia, G., Muma, N.A., Garcia, F., DonCarlos, L.L. (2002) Treatment of cycling female rats with fluoxetine induces desensitization of hypothalamic 5-HT_{1A} receptors with no change in 5-HT_{2A} receptors. Neuropharmacology, 43, 45-54.

142. Van Praag, H., Schinder, A.F., Christie, B.R., Toni, N., Palmer, T.D., Gage, F.H. (2002) Functional neurogenesis in the adult hippocampus. Nature, 415, 1030-1034.

143. Videbech, P., Ravnkilde, B.(2004) Hippocampal volume and depression: a meta-analysis of MRI studies. American Journal of Psychiatry, 161,11, 1957-1966.

144. Volmayr, B., Simonis, C., Weber, S., Gass, P., and Henn, F. (2003). Reduced cell proliferation in the dentate gyrus is not correlated with the development of helplessness. Biological Psychiatry 54, 1035-1040.

145. Vythilingam, M, Vermetten, E., Anderson, G.M., Luckenbaugh, E., Anderson, E.R., Snow, J., Staib, L.H., Charney, D.S., Bremner, J.D. (2004) Hippocampal volume, memory and cortisol status in major depressive disorder: Effects of treatment. Biol Psych. 56, 101-112.

146. Weber,C.C., Eckert, G.P., Muller, W.E. (2006) Effects of antidepressants on brain/plasma distribution of corticosterone. Neuropsychopharmacology, 31, 2443-48.

147. Weiss, J.M., Stone, E.A., Harrell, N. (1970) Coping behavior and brain norepinepherine level in rats. Journal of Comparative and Physiological Psychology,72, 153-60.

148. West, M.J., Slominanka, L., Gundersen, H.J.(1991) Unbiased stereological estimates of the total number of neurons in the subdivisions of the rat hippocampus using the optical franctionator. Anatomical Record, 231, 482-97.

149. Whittington, C. J., Dendall, T., Fonagy, P., Cottrell, D., Cotgrove, A., and Boddington, E. (2004). Selective serotonin reuptake inhibitors in childhood depression: systematic review of published versus unpublished data. The Lancet 363, 1341-1345.

150. Wilens, T. E., Biederman, J., Kwon, A., Chase, R., Greenberg, L., Mick, E., and Spencer, T. J. (2003). A systematic chart review of the nature of psychiatric adverse events in children and adolescents treated with selective serotonin reuptake inhibitors. Journal of child and adolescent psychopharmacology 13, 143-152.

151. Woolley, C.S. (1998) Estrogen-mediated structural and functional synaptic plasticity in the female rat hippocampus. Hormones and Behavior, 34,2,140-8.

152. Woolley CS, McEwen BS. (1993) Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. Journal of Comparative Neurology, 336,2, 293-306.

153. Woolley, C.S., Gould, E., Frankfurt, M, McEwen, B.S. (1990) Naturally occurring fluctuation in dendritic spine density on adult hippocampal pyramidal neurons. The Journal of Neuroscience, 10,12, 405-39.

154. Wyneken, U., Sandoval, M., Sandoval,S., Jorquera, F., Gonzalez, I., Vargas, F., Falcon, R., Moonari, M., Orrego, F. (2006) Clinically relevant doses of fluoxetine and

reboxetine induce changes in the TrkB content of central excitatory synapses. Neuropsychopharmacology, 31, 2415-23.

155. Young, S.N., Gauthier, S., Anderson, G.M., Purdy, W.C. (1980) Tryptophan, 5-hydroxyindoleacetic acid and indoleacetic acid in human cerebrospinal fluid: interrelationships and the influence of age, sex, epilepsy and anticonvulsant drugs. Journal of Neurology, Neurosurgery and Psychiatry, 43,5, 438-45.

156. Zazpe, A., Artaiz, I., Laeaga, L., Lucero, M.L., Orjales, A. (2006) Reversal of learned helplessness by selective serotonin reuptake inhibitors in rats is not dependent ofn 5-HT availability. Neuropharmacology, 52,3, 975-84.

157. Zhang, L., Ma,W., Barker,J.L., Rubinow, D.R. (1999) Sex differences in expression of serotonin receptors (subtypes 1A and 2A) in rat brain: A possible role of testosterone. Neuroscience, 94,1, 251-59.

TABLE 1

Results of 3 factor ANOVAs on pubescent and adult rats with treatment and sex as independent measures and number of labeled cells at 2hrs, 24hrs, and 28 days after BrdU injections as the dependent measures.

ANOVA	2hrs $F_{1,73}$ (adult) $F_{1,48}$ (puberty) (p- value)	24hrs $F_{1,32}$ (adult) $F_{1,22}$ (puberty) (p- value)	28d $F_{1,28}$ (adult) $F_{1,29}$ (puberty) (p- value)
Adult: treatment	14.13* (0.0003)	1.44 (0.238)	1.36 (0.259)
Adult: sex	4.13* (0.045)	3.98 (0.054)	33.91* (0.00003)
Adult: treatment x sex	4.55* (0.036)	5.29* (0.028)	4.33* (0.046)
Puberty: treatment	0.396 (0.532)	0.84 (0.370)	0.27 (0.607)
Puberty: sex	0.172 (0.680)	0.15 (0.706)	1.17 (0.287)
Puberty: treatment x sex	0.051 (0.822)	3.08 (0.093)	2.51 (0.123)

Asterisk denotes statistical significance of <0.05

TABLE 2

Linear regression statistics for BrdU labeled cell numbers at combined proliferation time points of 2hrs and 24 hrs.

Variable	b (Std. Error)	β (Std. Error)	t	p value
Treatment (1=fluoxetine)	430.51 (143.02)	0.13 (0.04)	3.00*	0.003
Sex (1=male)	185.01 (145.37)	0.06 (0.04)	1.27	0.20
Age (1=puberty)	1924.99 (145.18)	0.57 (0.04)	13.25*	0.0000
Sacrifice time (1=24hrs)	1965. 87 (153. 44)	0.55 (0.04)	12.81*	0.0000

$R^2 = 0.658$
Cases=191
$F(4,186)=89.66$, $p<0.05$

Asterisk denotes statistical significance of <0.05

TABLE 3

Linear regression statistics for BrdU labeled cell numbers at combined time points of 24hrs and 28 days.

Variable	b (Std. Error)	β (Std. Error)	t	p value
Treatment (1=fluoxetine)	210 (179.83)	0.067 (0.06)	1.17	0.245
Sex (1=male)	428.23 (179.83)	0.137 (0.06)	2.38*	0.018
Age (1=puberty)	2217.44 (180.70)	0.708 (0.06)	12.27*	0.0000
Sacrifice time (1=24hrs)	-1046.15 (180.30)	-0.335 (0.06)	-5.80*	0.0000

$R^2 = 0.59618$
Cases=127
$F(4,122)=45.03$, $p<0.05$

Asterisk denotes statistical significance of <0.05

FIGURE LEGENDS

Figure 1. Schematic drawing of injection schedules for experiment 1, 2, and 3. In all experiments rats were injected with 5mg/kg of fluoxetine or saline vehicle for 14 days. One day after the end of treatment subjects were injected with 200mg/kg of BrdU. In experiment 1 rats were sacrificed 2hrs after BrdU injection. In experiment 2 rats were sacrificed 24 hrs after BrdU injection. In experiment 3 rats were sacrificed 28 days after BrdU injection. Pubescent rats began injections of fluoxetine at 24-26 days of age, prior to the beginning of puberty, adult rats began injections of fluoxetine when they were older than 60 days but younger than 90 days of age.

Figure 2. Photomicrographs of BrdU labeled cells in the dentate gyrus of the hippocampus 2hrs, 24hrs, and 28 days after injection. A) Photomicrographs (40x) of BrdU labeled cells stained with peroxidase methods in male and female rats during adulthood and puberty treated with fluoxetine or saline and sacrificed 2hrs after BrdU injection. B) Photomicrographs (40x)of BrdU labeled cells stained with peroxidase methods in male and female rats during adulthood and puberty treated with fluoxetine or saline and sacrificed 24hrs after BrdU injection. A) Photomicrographs (40x) of BrdU labeled cells stained with peroxidase methods in male and female rats during adulthood and puberty treated with fluoxetine or saline and sacrificed 28 days after BrdU injection.

Figure 3. Photomicrographs (40x) of BrdU and NeuN co-labeled cells stained with immunofluorescent methods taken from rats sacrificed 28 days after BrdU injections. Arrows point to BrdU labeled cells. A) Double labeled cells from adult saline

and fluoxetine treated male rats. B) Double labeled cells from adult saline and fluoxetine treated female rats. C) Double labeled cells from pubescent saline and fluoxetine treated male rats. D) Double labeled cells from pubescent saline and fluoxetine treated female rats.

Figure 4. The effects of fluoxetine treatment on cell proliferation and neurogenesis in male and female, pubescent and adult rats sacrificed 2hrs, 24hrs and 28 days after BrdU injection. Graphs depict the mean ± SEM number of BrdU labeled cells. One asterisk denotes main effects, two asterisks denote an interaction. A) Chronic treatment with fluoxetine significantly increased cell proliferation 2hrs after BrdU injection in adult male rats, but not adult female rats. B) Chronic treatment with fluoxetine did not increase cell proliferation 2hrs after BrdU injection in pubescent male or female rats. C) Chronic treatment with fluoxetine significantly increased cell proliferation 24hrs after BrdU injection in adult male rats, but did not increase cell proliferation in adult female rats. D) Chronic treatment with fluoxetine did not significantly increase cell proliferation 24hrs after BrdU injection in pubescent male or female rats. E) Chronic treatment with fluoxetine increased neurogenesis in adult male rats sacrificed 28 day after BrdU injection. Treatment did not increase neurogenesis in adult females, females had fewer new cells than males at this time point. F) Chronic treatment with fluoxetine did not increase neurogenesis 28 days after BrdU injection in male or female rats during puberty.

Figure 5. The effects of chronic fluoxetine treatment on cell proliferation measured at 2hrs after BrdU injection during the stages of the estrous cycle. Graphs depict the

mean ± SEM number of BrdU labeled cells. One asterisk denotes main effects, two asterisks denote an interaction. A) Chronic fluoxetine treatment did not increase cell proliferation at any stage of the estrous cycle in adult females, nor were there any effects of the estrous cycle on cell proliferation. B) Chronic treatment with fluoxetine did not increase cell proliferation at any stage of the pubescent estrous cycle, nor were there any effects of the stage of the cycle on cell proliferation.

Figure 6. The effects of fluoxetine treatment on the number of new neurons co-labeled with BrdU and NeuN in rats sacrificed 28 days after BrdU injection. Graphs depict the mean ± SEM number of double labeled cells. One asterisk denotes main effects, two asterisks denote an interaction. A) Chronic treatment with fluoxetine increased the number of new neurons in adult male rats, but not adult female rats. B) Chronic treatment with fluoxetine did not increase the number of new neurons in male or female rats during puberty.

Figure 7. The effects of chronic fluoxetine treatment on cell fate measured by the percentage of double labeled cells in rats sacrificed 28 days after BrdU injection. Graphs depict the mean ± SEM percentage of double labeled cells. A) Chronic treatment with fluoxetine did not alter the percentage of cells that were co-labeled with BrdU and NeuN in adult males or females. B) Chronic treatment with fluoxetine did not alter the percentage of cells that were co-labeled with BrdU and NeuN in pubescent males or females.

Figure 8. During puberty male and female rats produce more new cells than adults 2hrs, 24hrs and 28 days after BrdU injection. Graphs depict the mean ± SEM number of BrdU labeled cells in saline treated pubescent and adult rats. One asterisk denotes main effects, two asterisks denote an interaction. A) Male and female pubescent rats produce more new cells than adults sacrificed 2hrs after BrdU injection. B) Male and female pubescent rats produce more new cells than adults sacrificed 24hrs after BrdU injection. C) Male and female pubescent rats produce more new cells than adults sacrificed 28 days after BrdU injection.

Figure 9. The effects of chronic treatment with fluoxetine on circulating levels of corticosterone measured 1 day or 29 days after the cessation of treatment. Graphs depict the mean ± SEM of circulating corticosterone levels (ng/ml). One asterisk denotes main effects, two asterisks denote an interaction. A) Chronic treatment with fluoxetine did not alter circulating levels of corticosterone in adult male or female rats. B) Chronic treatment with fluoxetine did not alter circulating levels of corticosterone in male or female rats during puberty; pubescent females had lower levels of circulating corticosterone than males regardless of treatment. C) Treatment with fluoxetine did not alter circulating levels of corticosterone after a 29 day wash out period in adult male or female rats. D) Chronic treatment with fluoxetine during puberty decreased corticosterone concentrations in pubescent females during adulthood after a 29 day wash out period. Corticosterone concentrations in adult males and saline treated females were not altered.

Figure 10. The effects of chronic fluoxetine treatment on circulating levels of estradiol in pubescent and adult female rats 1 day and 29 days after the cessation of treatment. Graphs depict the mean ± SEM circulating levels of estradiol (pg/ml). One asterisk denotes main effects, two asterisks denote an interaction. A) Chronic fluoxetine treatment did not alter circulating levels of estradiol during any stage of the estrous cycle in adult female rats. Adult female rats during proestrus had significantly higher levels of estradiol than females at any other stage of the cycle. B) During puberty fluoxetine treatment decreased estradiol concentrations during the estrus stage of the cycle. C) After a 29 day wash out period saline treated adult females had higher estradiol concentrations than adult females treated with fluoxetine, and pubescent females of both treatment groups.

Figure 11. The effects of fluoxetine treatment on circulating levels of testosterone 1 day and 29 days after fluoxetine treatment in pubescent and adult male and female rats. Graph depicts the mean circulating levels of testosterone (ng/dl). One asterisk denotes main effects, two asterisks denote an interaction.(A) Chronic fluoxetine did not alter circulating levels of testosterone in adult male or female rats sacrificed 1 day after the cessation of treatment. Males had significantly higher levels of testosterone than females. B) Chronic fluoxetine did not alter circulating levels of testosterone in male or female pubescent rats 1 day after cessation of treatment. Male pubescent rats had significantly higher testosterone levels than pubescent females. C) Chronic fluoxetine treatment did not alter testosterone levels in adult male or female rats sacrificed 29 days after the cessation of treatment. Adult males had significantly higher levels of

testosterone than females. D) Chronic fluoxetine treatment did no significantly alter testosterone levels in pubescent male and female rats sacrificed 29 days after cessation of treatment. Male pubescent rats had significantly higher levels of testosterone than females.

Figure 1

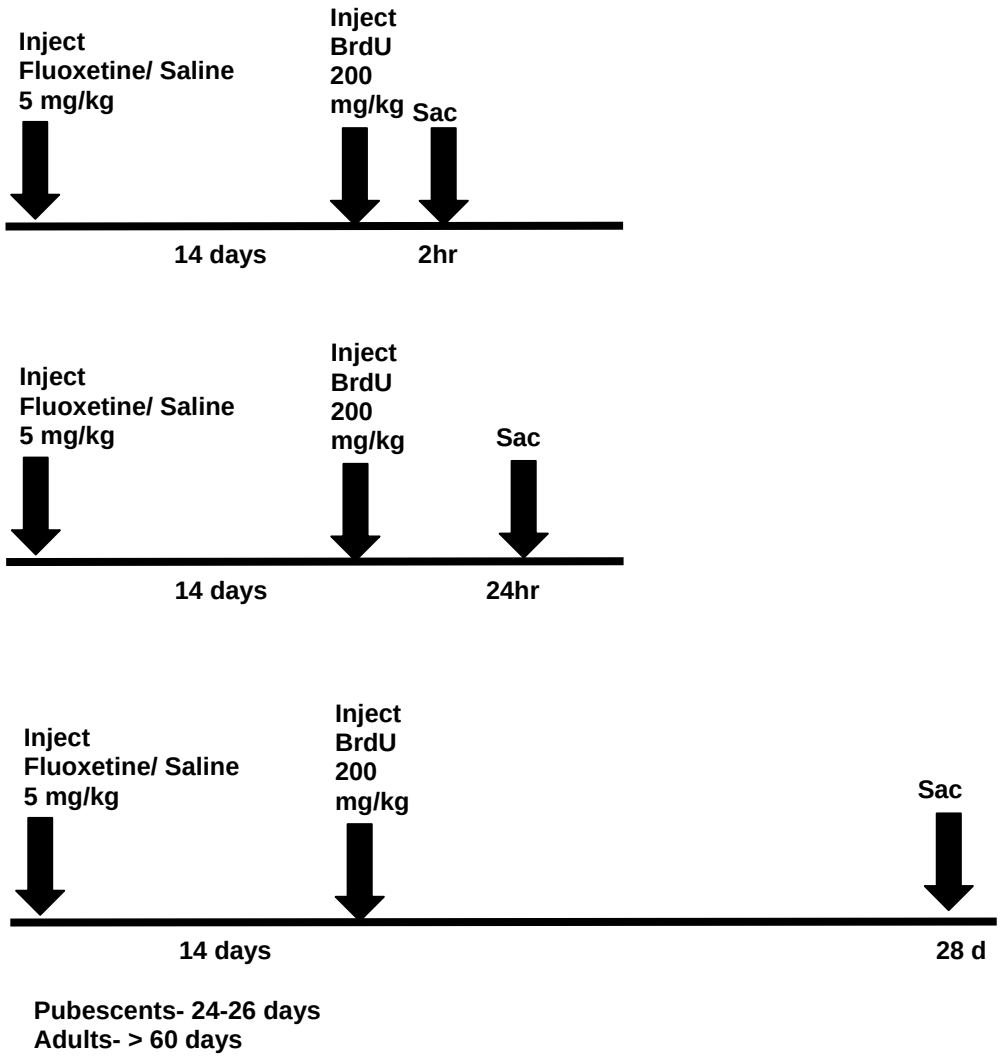

Inject
Fluoxetine/ Saline
5 mg/kg
Inject
BrdU
200
mg/kg
Sac
14 days
2hr
Inject
Fluoxetine/ Saline
5 mg/kg
Inject
BrdU
200
mg/kg
Sac
14 days
24hr
Inject
Fluoxetine/ Saline
5 mg/kg
Inject
BrdU
200
mg/kg
Sac
14 days
28 d
Pubescents- 24-26 days
Adults- > 60 days

Figure 2

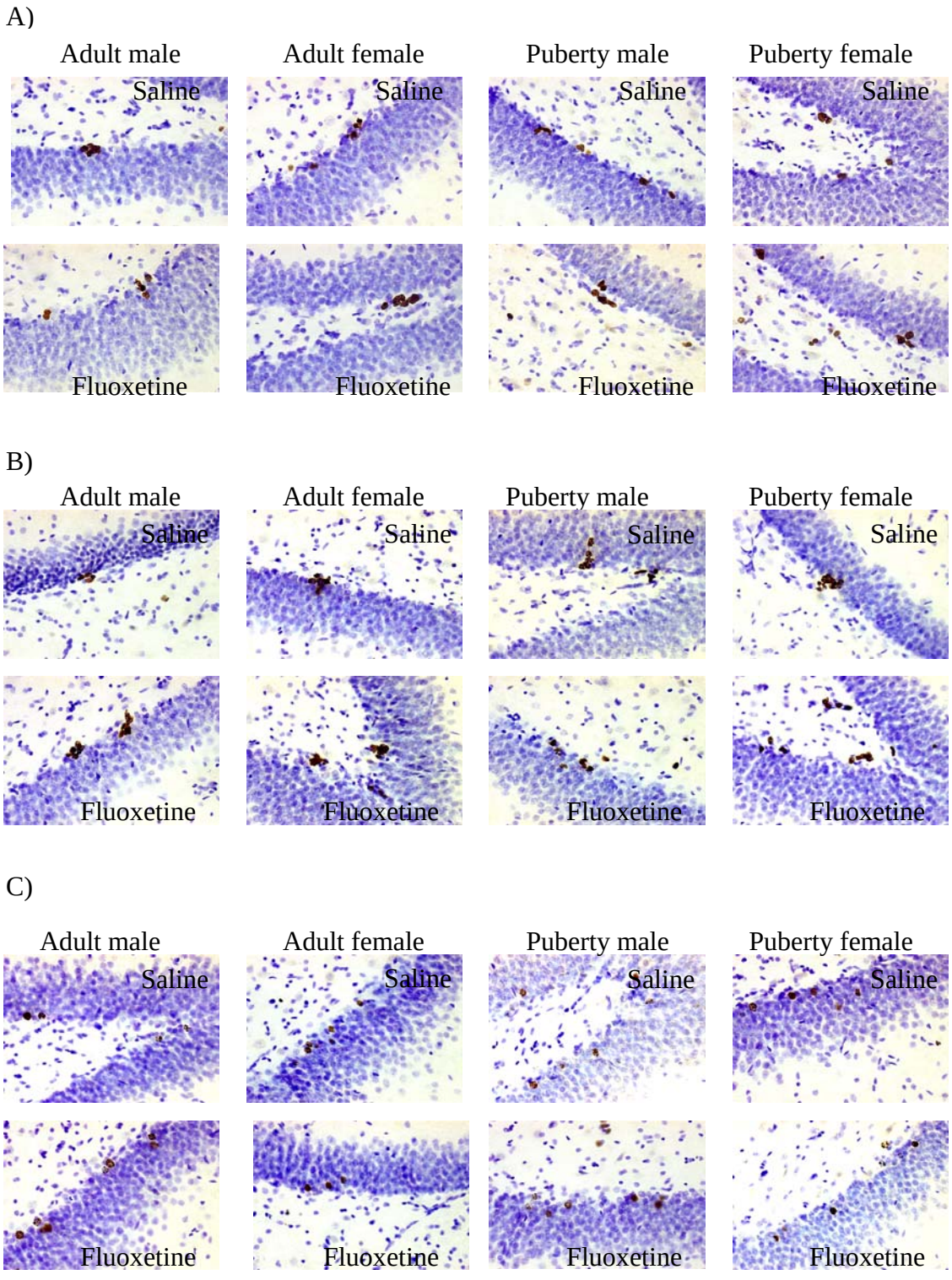
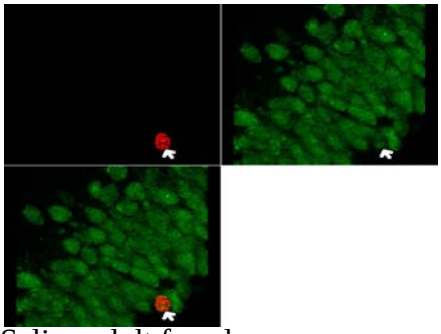
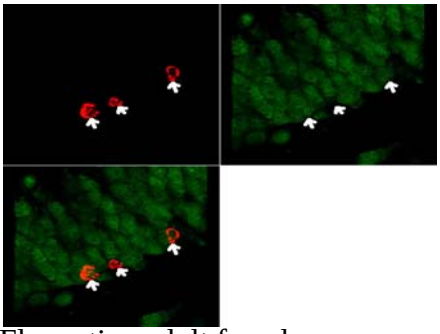

A)
Adult male
Adult female
Puberty male
Puberty female
Saline
Saline
Saline
Saline
Fluoxetine
Fluoxetine
Fluoxetine
Fluoxetine
B)
Adult male
Adult female
Puberty male
Puberty female
Saline
Saline
Saline
Saline
Fluoxetine
Fluoxetine
Fluoxetine
Fluoxetine
C)
Adult male
Adult female
Puberty male
Puberty female
Saline
Saline
Saline
Saline
Fluoxetine
Fluoxetine
Fluoxetine
Fluoxetine

Figure 3

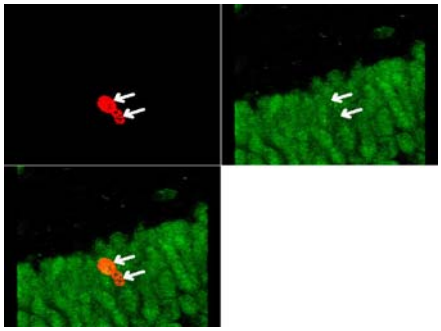
A) Saline adult male



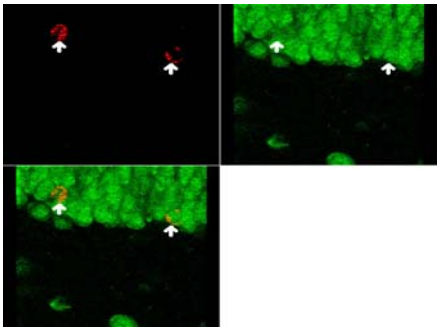
Fluoxetine adult male



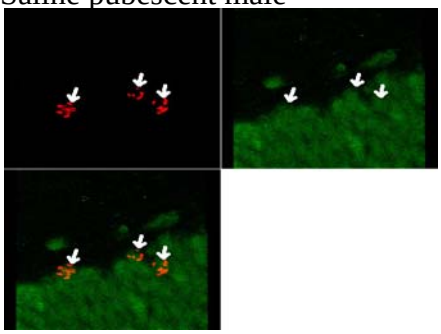
B) Saline adult female



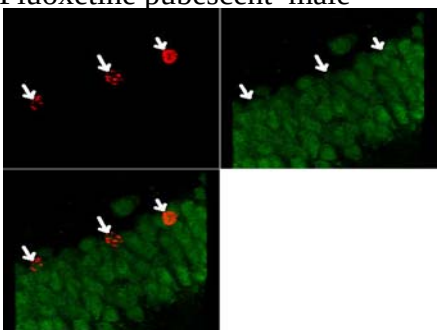
Fluoxetine adult female



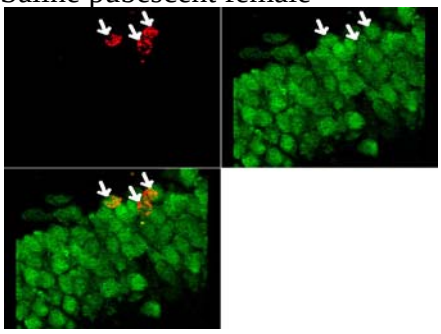
C) Saline pubescent male



Fluoxetine pubescent male



D) Saline pubescent female



Fluoxetine pubescent female

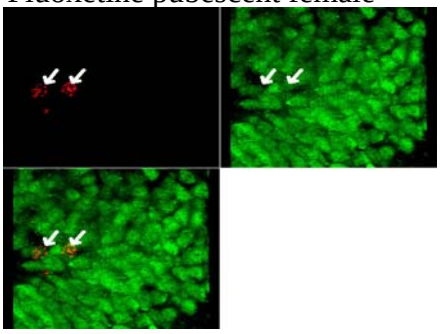


Figure 4

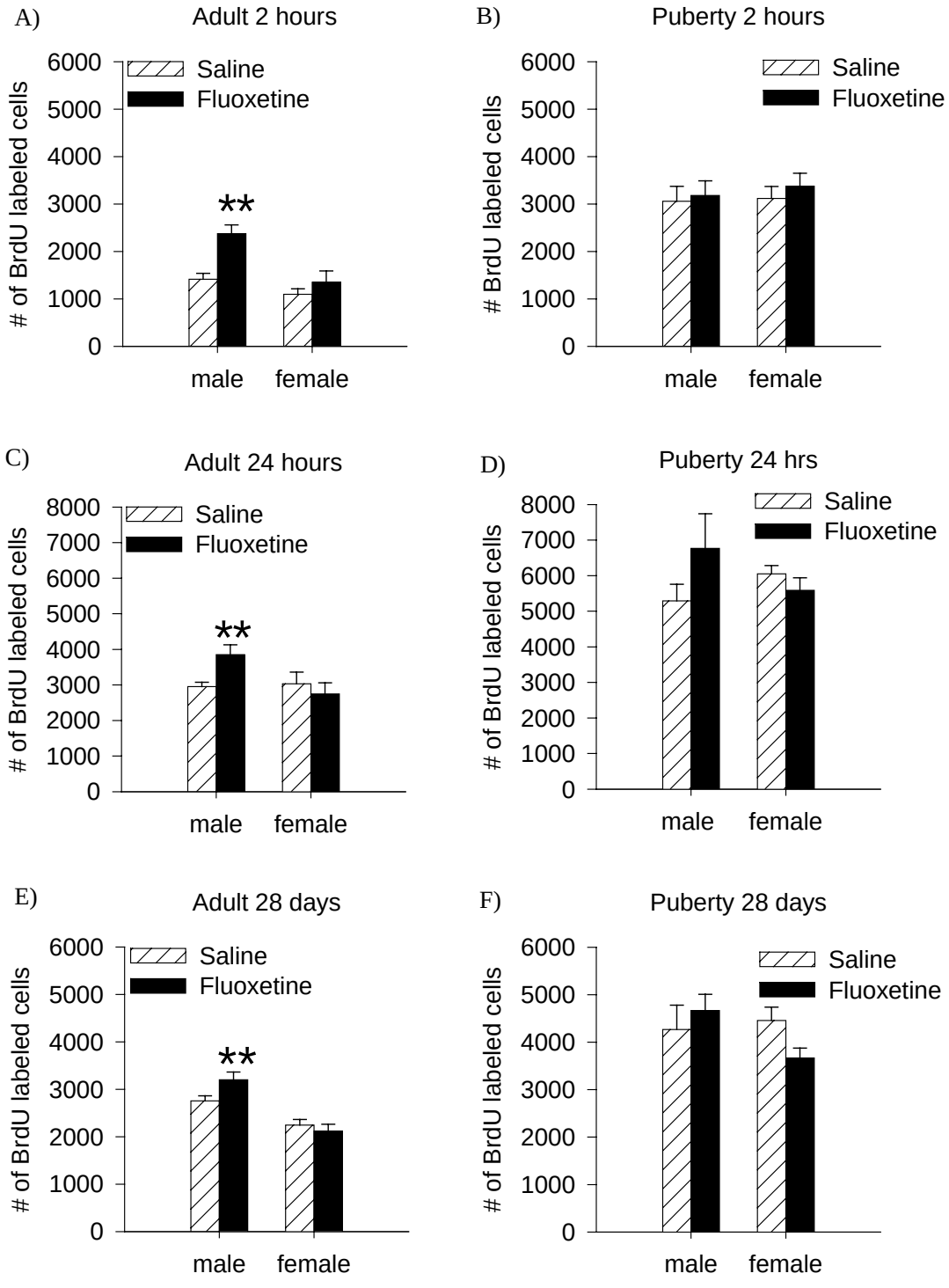

A)
Adult 2 hours
B)
Puberty 2 hours
C)
Adult 24 hours
D)
Puberty 24 hrs
E)
Adult 28 days
F)
Puberty 28 days
Saline
Fluoxetine
# of BrdU labeled cells
male
female
**

Figure 5

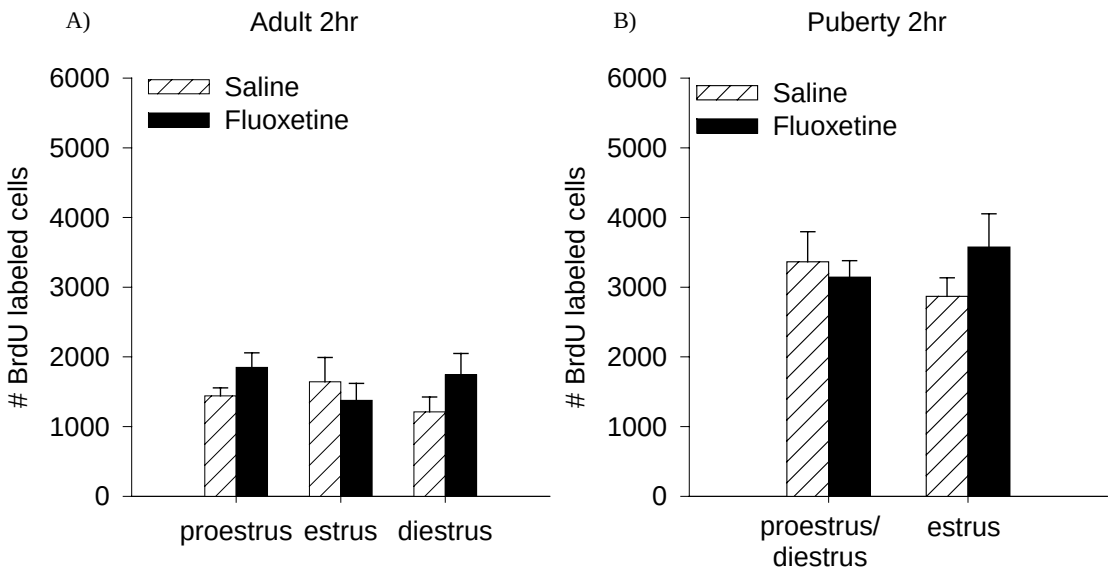

A)
Adult 2hr
Saline
Fluoxetine
BrdU labeled cells
6000
5000
4000
3000
2000
1000
0
proestrus
estrus
diestrus
B)
Puberty 2hr
Saline
Fluoxetine
BrdU labeled cells
6000
5000
4000
3000
2000
1000
0
proestrus/
diestrus
estrus

Figure 6

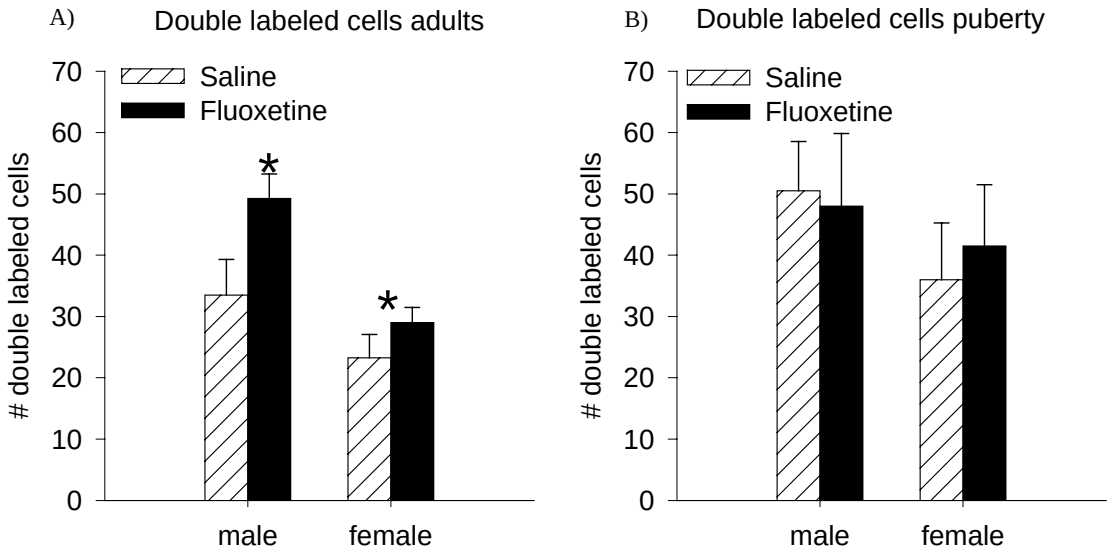

A)
Double labeled cells adults
Saline
Fluoxetine
double labeled cells
70
60
50
40
30
20
10
0
*
*
male
female
B)
Double labeled cells puberty
Saline
Fluoxetine
double labeled cells
70
60
50
40
30
20
10
0
male
female

Figure 7

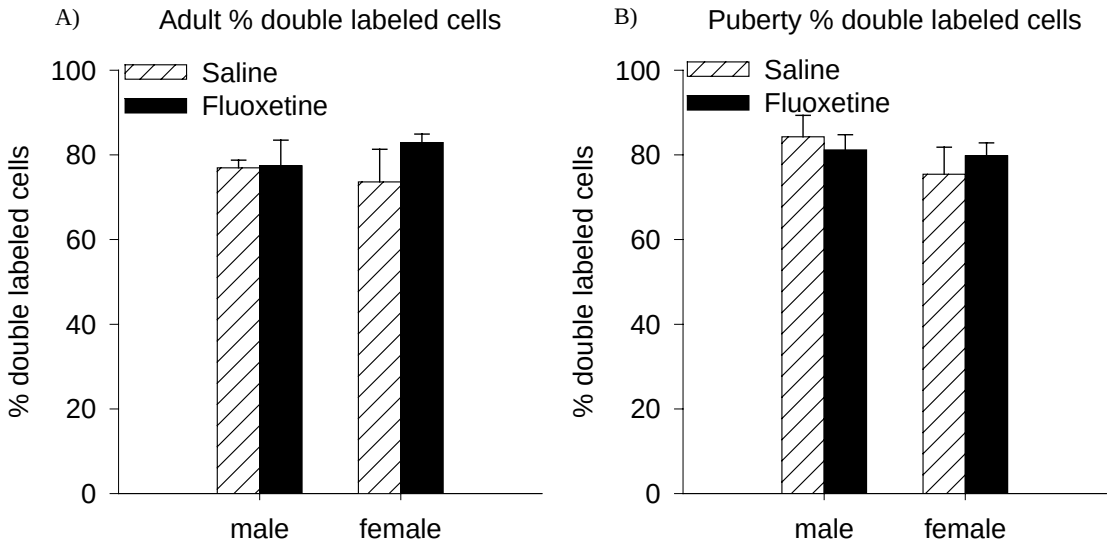

A)
Adult % double labeled cells
Saline
Fluoxetine
% double labeled cells
100
80
60
40
20
0
male
female
B)
Puberty % double labeled cells
Saline
Fluoxetine
% double labeled cells
100
80
60
40
20
0
male
female

Figure 8

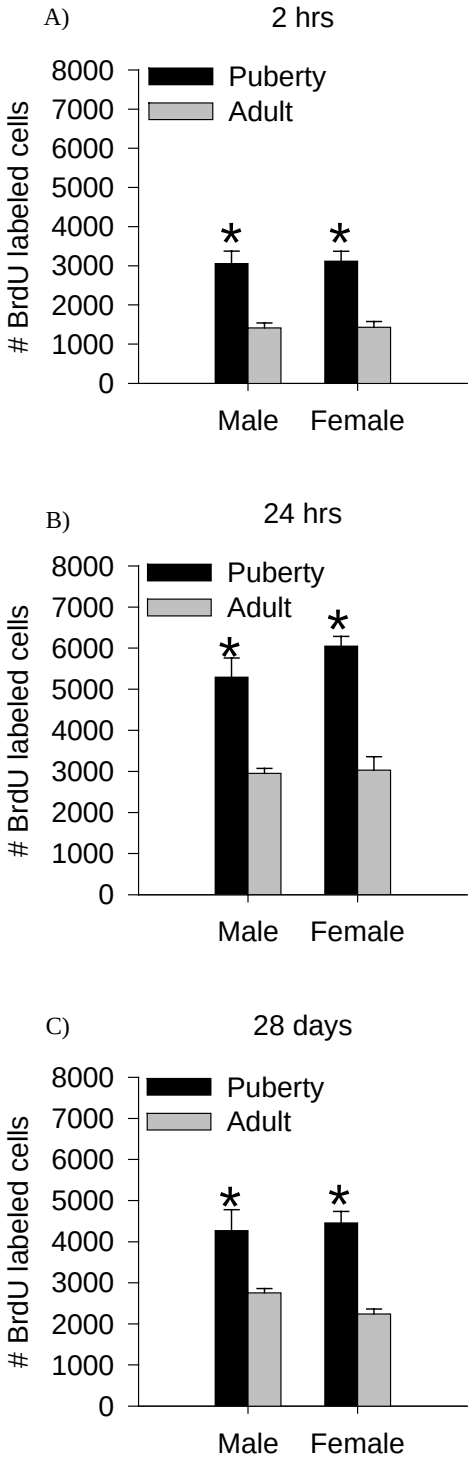

A)
2 hrs
Puberty
Adult
BrdU labeled cells
8000
7000
6000
5000
4000
3000
2000
1000
0
*
*
Male
Female
B)
24 hrs
Puberty
Adult
BrdU labeled cells
8000
7000
6000
5000
4000
3000
2000
1000
0
*
*
Male
Female
C)
28 days
Puberty
Adult
BrdU labeled cells
8000
7000
6000
5000
4000
3000
2000
1000
0
*
*
Male
Female

Figure 9

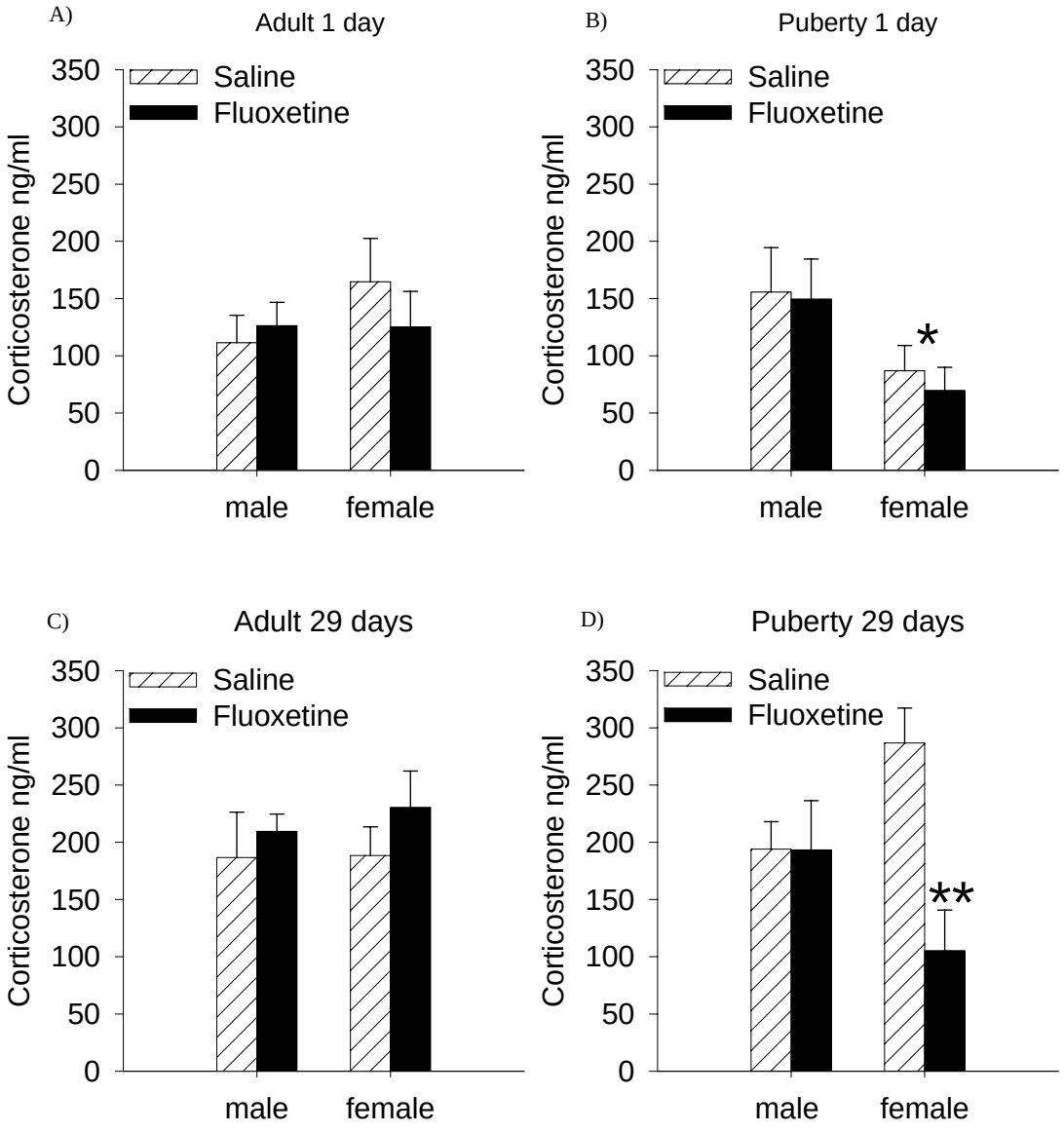

A)
Adult 1 day
Saline
Fluoxetine
Corticosterone ng/ml
350
300
250
200
150
100
50
0
male
female
B)
Puberty 1 day
Saline
Fluoxetine
Corticosterone ng/ml
*
male
female
C)
Adult 29 days
Saline
Fluoxetine
Corticosterone ng/ml
male
female
D)
Puberty 29 days
Saline
Fluoxetine
Corticosterone ng/ml
**
male
female

Figure10

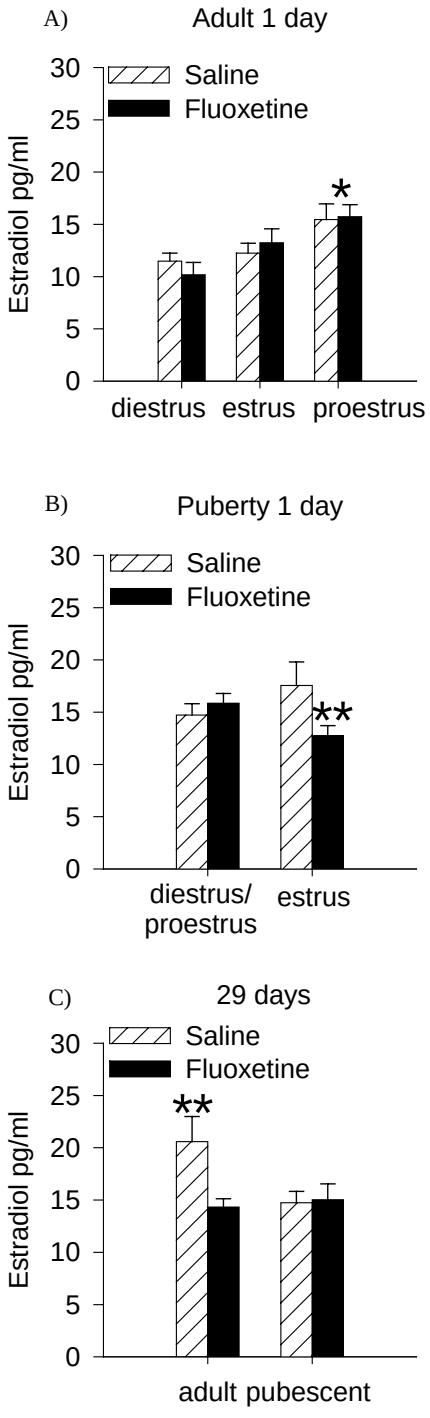
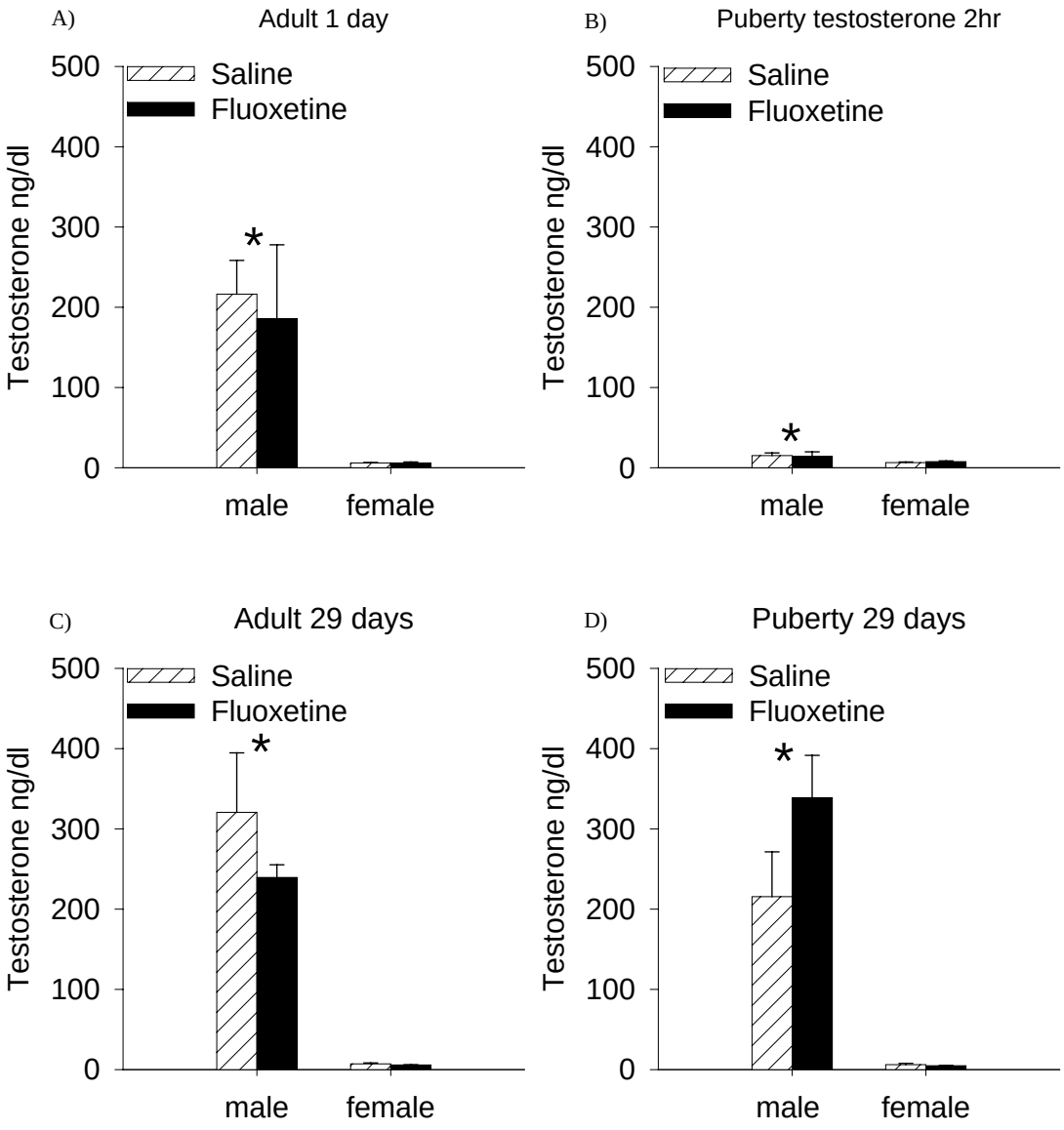

A)
Adult 1 day
Saline
Fluoxetine
Estradiol pg/ml
30
25
20
15
10
5
0
*
diestrus
estrus
proestrus
B)
Puberty 1 day
Saline
Fluoxetine
Estradiol pg/ml
30
25
20
15
10
5
0
**
diestrus/
proestrus
estrus
C)
29 days
Saline
Fluoxetine
Estradiol pg/ml
30
25
20
15
10
5
0
**
adult
pubescent

Figure 11


A)
Adult 1 day
B)
Puberty testosterone 2hr
C)
Adult 29 days
D)
Puberty 29 days
Saline
Fluoxetine
Testosterone ng/dl
500
400
300
200
100
0
male
female
*

CIRRICULUM VITAE

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Hodes, G.E., Shors, T.J. (2006). Learning during middle age: A resistance to stress? *Neurobiology of Aging*, Epub September 11, in press

Hodes, G.E., Shors, T.J. (2005) Distinctive stress effects on learning during puberty. *Hormones and Behavior, 48,2,*163-171.

Hodes, G.E, Shors, T.J. Ovarian hormones interact with stressful experience to modify learning during puberty and adulthood. In preparation.

Hodes, G.E., Shors, T.J. Prozac & Puberty: The effects of fluoxetine treatment on neurogenesis in the pubescent hippocampus. In preparation.