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Prozac during puberty: Distinctive effects on neurogenesis as a function of age and sex

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Abstract

Neurogenesis is a possible substrate through which antidepressants alleviate symptoms of depression. In adult male rodents and primates, chronic treatment with fluoxetine increases neurogenesis in the hippocampal formation. Little is known about the effects of the antidepressant on neurogenesis during puberty or in female animals at any age. Therefore we examined the effects of chronic fluoxetine treatment on cell proliferation and survival in male and female rats during puberty and adulthood.

Adult and peri-pubescent male and female rats were treated chronically with fluoxetine (Prozac-5 mg/kg) or saline. Subsequently rats received a single injection of 5-bromo-2'-deoxyuridine (BrdU; 200 mg/kg) to label DNA synthesis. Rats were sacrificed 2 hrs, 24 hrs, or 28 days after BrdU injection to examine cell proliferation, survival and cell fate. Fluoxetine increased cell proliferation in adult male rats but not in peri-pubescent males or female rats of any age or stage of the estrous cycle. Treatment did not alter the number of surviving cells in the male hippocampus but decreased survival in the female hippocampus. Thus, fluoxetine has distinctive effects on neurogenesis as a function of age and sex. Circulating levels of the stress hormone corticosterone were also examined. Treatment of female rats with fluoxetine during puberty decreased circulating levels of corticosterone as adults, even in the absence of the drug suggesting disruption of maturation of the hypothalamic-pituitary-adrenal axis.

Keywords

Corticosterone; adolescence; rats; antidepressants; cell proliferation; cell survival

Currently there is a great deal of controversy surrounding the administration of antidepressants to adolescents. Initial research indicated that treatment of adolescents with the selective serotonin re-uptake inhibitor (SSRI) class of antidepressants resulted in an increased risk of suicidal thoughts (FDA). Since this warning was issued, however, adolescent suicide rates have increased and many in the health care profession believe that this increase is the direct result of the decrease in prescriptions of antidepressants to adolescents (Kuehn, 2007). While many studies have examined the behavioral and emotional impacts of antidepressants in human subjects, few have examined the biological impact of these drugs during this developmental period in animals.

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One model of adolescent development is the peri-pubescent rat (post natal days [PND] 24–56) covering the transition from the juvenile period through puberty in both sexes (Ojeda and Urbanski, 1994). Onset of puberty in the rat can be physically determined in females by visual inspection of the opening of the vaginal canal and first estrus, and in males by examination of balano-preputial separation (Ojeda and Urbanski, 1994). During the peri-pubescent period there are differences from adults in learning ability and behavioral stress response (Hodes and Shors, 2005), neurotransmitter release (Choi et al., 1997), neurogenesis (Heine et al., 2004, McDonald and Wojtowicz, 2005) and the hormonal stress response (Romeo et al., 2004a, Romeo et al., 2004b).

To determine whether antidepressants differently affect neuroplasticity in the peri-pubescent brain, the following experiments were conducted with fluoxetine, which is the only antidepressant currently approved for pediatric depression in the United States (FDA). The current study compared the effects of fluoxetine treatment on cell proliferation, survival and phenotype during puberty and adulthood. In adult male rodents and primates, chronic but not acute treatment with a variety of antidepressants increases neurogenesis in the dentate gyrus of the hippocampus (Malberg et al., 2000, Kodama et al., 2004, Encinas et al., 2006, Perera et al., 2007). Therefore, neurogenesis was used as the dependent measure in this study as this process has been implicated in the etiology of depression (Duman et al., 2001, Sapolsky, 2004). Circulating levels of corticosterone were also examined to determine whether fluoxetine treatment differently affects stress hormones in males and females at either age. Currently there are discrepancies in the literature surrounding the effects of fluoxetine treatment on basal levels of the stress hormones. In adult male mice, 14 days of fluoxetine treatment (10 mg/kg) increased plasma and brain corticosterone (Weber et al., 2006). Whereas in humans, four weeks of fluoxetine treatment decreased cortisol levels in both sexes with the larger decrease occurring in females (Bano et al., 2004). Additionally, early life exposure to fluoxetine may alter maturation of the Hypothalamic Pituitary Adrenal (HPA) axis as it increases measures of anxiety and depression-like behaviors in adult mice (Ansorge *et al.*, 2004).

Experimental Procedures

Animals

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. Sprague-Dawley rats were bred on premises from stock obtained from Harlan Sprague-Dawley (Indianapolis, IN) and maintained on *ad libitum* water and laboratory chow with a 12:12 light/dark cycle. Peri-pubescent rats were weaned between PND 21–23 and thereafter housed singly. The adults were weaned between PND 26–28 and group housed until 60 days of age, at which time they were individually housed. Animals were weaned at different ages and housed differently so that all animals would have 3–5 days of single housing prior to the start of injections. Peri-pubescent rats were 24–26 days of age when the injections began and 38–40 days of age when they ended. These ages were chosen because they include the transition from the juvenile period (PND 21–35 males/PND 21–32 females) through puberty (PND 35–56 males/PND 33–56 females) (Ojeda and Urbanski, 1994). The adult animals were at least 63–5 days of age when the injections began and no more than 90 days when they ended.

Animals were examined for physiological indicators of puberty using first ovulation/vaginal opening in the female and balano-preputial separation in the male. Stages of the estrous cycle were determined by daily vaginal swabs as previously described (Wood and Shors, 1998). All adult female rats included in the study had a regular 4–5 day estrous cycle. Peri-pubescent

female rats have an irregular cycle (Hodes and Shors, 2005) and therefore no females in that age range were excluded on the basis of their estrous stage.

Experiment 1

The number of cells generated within 2 hr of the BrdU injection was used to indicate the extent of DNA synthesis without the contribution of migration or cell death (Nowakowski *et al.*, 1989) (Fig. 1a). Adult (n= 14 per treatment) and peri-pubescent (n= 8–15 per treatment) male rats were injected with either saline or fluoxetine for 14 days. Cell counts were determined for adult females during each stage of the estrous cycle; proestrus (n= 8–12 per treatment group), estrus (n= 7–8 per treatment group) and diestrus (7–8 per treatment group). Female peri-pubescent rats have an irregular estrous cycle which alternates between a stage with characteristics of both proestrus/diestrus and estrus (Hodes and Shors, 2005). Therefore, cell counts were generated for peri-pubescent females during estrus (7–8 per treatment group) and diestrus/proestrus (n= 7–8 per treatment group). In a subset of these groups, blood was collected for radioimmunoassay of corticosterone concentrations the day after treatment ended (n= 90, 5 per group including each stage of the estrous cycle).

Injections

Rats received intraperitoneal injections (i.p.) of the selective serotonin reuptake inhibitor (SSRI) fluoxetine (5 mg/kg, gift of Eli Lilly) or a weight-based equivalent dose of saline (0.9%) for a minimum of 14 days and a maximum of 18 days. In order to examine the number of cells generated in each stage of estrous, some adult females received more than 14 days (but no more than 18 days) of injections. Separate 2 way (drug treatment $\times$ length of injection period) ANOVAs were performed on cell counts from peri-pubescent and adult females to determine that elongation of treatment did not alter cell proliferation. Fluoxetine was injected at 5 mg/kg because this dose increases cell proliferation in adult male rats (Malberg *et al.*, 2000, Kodama *et al.*, 2004) alters stress induced behavior in adult female rats (Leuner *et al.*, 2004) and is on the high end of the spectrum of a clinically relevant dose in humans (Alvarez *et al.*, 1998, Wyneken *et al.*, 2006). One day after the final fluoxetine/saline injection animals received a single i.p. injection of BrdU (200 mg/kg; Sigma) a thymidine analog which is incorporated into the DNA of cells during the S phase of the cell cycle (Nowakowski *et al.*, 1989). Injections of BrdU were given between 11 am and 2:30pm.

Sacrifice and perfusion

Rats were sacrificed two hours after BrdU injection. Animals were deeply anesthetized with a lethal dose of 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 post-fixed in 4% paraformaldehyde for a minimum of 48 hr, and then transferred to PBS (PH=7.3).

Immunohistochemistry methods

Coronal sections (40 μ m) 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, 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, Franklin Lakes, NJ) 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 Vector Laboratories, Burlingame, CA). After another series of rinses, sections were incubated in avidin–biotin– horseradish peroxidase (Vector

Laboratories, Burlingame, CA) for 1 hour, and then stained with diaminobenzidine (Sigma-Aldrich, St. Louis, MO) 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). Positive and negative control sections were included with each batch of slides stained for BrdU. Positive controls were sections taken from animals who had previously displayed staining. Negative controls underwent the secondary treatments but were incubated in PBS rather than primary antibody overnight.

Slides were coded prior to quantitative analysis and cells were counted blind to the experimental conditions. Cells were visualized under 1000 $\times$ (100 $\times$ oil immersion objective with a 10 $\times$ 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 was 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 then multiplied by 24 (number of intervening slices $\times$ 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.

Radioimmunoassay

Cardiac blood was collected with heparin (0.01ml) at the time of sacrifice (1pm–4:30pm) and centrifuged for 20 min at 3,000 rpm. Plasma aliquots were stored frozen until analysis. Circulating levels of corticosterone was analyzed using a solid-phase radioimmunoassay system (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA) The assay sensitivity for corticosterone was 5.7ng/ml. Intra-assay variability and inter-assay variability for corticosterone was 4.3% and 5.8% respectively.

Experiment 2

The second experiment was conducted to determine how many new cells were generated 24 hrs after a single BrdU injection. This time point was used to determine the number of cells that underwent DNA synthesis, divided, and produced progeny, rather than exiting the cell cycle to die (Cameron and McKay, 2001). As in the first experiment, peri-pubescent rats were 24–26 days of age when the injections began and 38–40 days when they ended. Adults were at least 60 days old at the onset of injections and no more than 90 days old when they ended. Adult males (n=9–11 per treatment), peri-pubescent males (n=5–7 per treatment), and peri-pubescent females (n= 6–7 per treatment) received daily injections of fluoxetine (5 mg/kg) or saline for 14 days (Fig. 1b). Rats were injected with BrdU (200 mg/kg) one day after the last fluoxetine/saline injection. The adult females (n= 8 per treatment group) were in proestrus at the time of BrdU injection. Because of this, some adult females received more than 14 days of treatment with no more than 18 days. They were then sacrificed 24 hrs after BrdU injection. Stages of the estrous cycle were tracked in peri-pubescent females but no attempt was made to categorize by stage of cycle. Brains were processed for peroxidase staining as described in the methods for experiment 1.

Experiment 3

The third experiment was conducted to determine whether new cells that were generated during fluoxetine treatment were present 28 days after the BrdU injection. This number indicates how many cells survived and differentiated into neurons (Dayer *et al.*, 2003) (Fig. 1c). As before, peri-pubescent rats were 24–26 days at the beginning of treatment and 38–40 days when it ended. Adults were at least 60 days old when it began and no more than 90 days old when it

ended. Adult males (n= 8 per treatment); adult females (n=7–9 per treatment); peri-pubescent males (n=8–9 per treatment); and peri-pubescent females (n=7–8 per treatment group) were treated with fluoxetine (5 mg/kg) or a weight-based saline control for 14 days. Animals were injected with BrdU (200 mg/kg) on day after the end of treatment and sacrificed 28 days later. Stages of the estrous cycle were tracked in both adult and peri-pubescent females but no attempt was made to categorize by stage of cycle. Cells were labeled with peroxidase methods as previously described in experiment 1. Trunk blood was collected from a subset of rats (n=40, 5 per group) and analyzed using radioimmunoassay.

Immunofluorescence labeling and microscopy

To assess cell fate, sections were randomly selected from a subset of the animals (n= 16, 4 per group) and double labeled with BrdU and neuronal nuclear antigen (NeuN). 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 90 min, 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). 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 all BrdU labeled 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 um thick sections through the optical stack and verification was performed through examination of cells in the orthogonal planes.

Statistical analysis

Analyses were performed using the statistical program Statistica (StatSoft Inc, Tulsa, OK). The effects of drug treatment (saline/fluoxetine) on cell counts and hormone levels in males and females of both ages were determined using 3 way ANOVAS (drug treatment $\times$ sex $\times$ age). Significant interactions were examined using Newman-Keuls. Planned comparisons were used to detect treatment effects within each sex and age. Separate 2 way ANOVAs (drug treatment $\times$ stage of cycle) were run in adult and peri-pubescent females at the 2 hr time point because the stages of the estrous cycle differ between ages. Additional 2 way ANOVAs (drug treatment $\times$ number of days of injections) were performed on data from the females to detect whether there were any effects of elongating fluoxetine treatment to label cells in the correct stage of the 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 were transformed, arcsin values were analyzed with 3 way ANOVA comparing treatment effects on both sexes for each age.

Results

Experiment 1

Examination of the number of cells labeled in 2 hrs was used as a measure of cell proliferation, to determine the number of cells undergoing DNA synthesis without migration or cell death (Nowakowski *et al.*, 1989). Omnibus ANOVA indicated that fluoxetine treatment increased cell proliferation when data was collapsed across groups ($F_{1,124} = 5.20, p < 0.05$). Overall, pubescent animals possessed more BrdU labeled cells in their hippocampus than did adults ($F_{1,124} = 84.76, p < 0.001$). However, analysis with planned comparisons revealed that

treatment with fluoxetine over 14 days did not increase the number of BrdU-labeled cells when animals were treated during puberty. This was the case for both males ($p = 0.68$) and females ($p = 0.59$). This is in contrast to adult males treated with fluoxetine for 14 days (Fig. 2a). They expressed a 1.6 fold increase ($p = 0.007$), with numbers similar to those previously reported (Malberg et al., 2000, Kodama et al., 2004). Surprisingly, fluoxetine did not increase the number of BrdU labeled cells in females ($p = 0.34$). Even when separated according to stage of estrus, there was no differences in cell proliferation in the hippocampus of peri-pubescent females ($F_{1,26} = 2.07, p > 0.05$) (Fig 2b) or adult females ($F_{2,44} = 1.27, p > 0.05$) (Fig. 2c). In order to associate changes in proliferation with stages of estrus, some female rats had undergone more than 14 days of fluoxetine treatment. But even under this longer treatment protocol, there was no observable effect of fluoxetine treatment on the number of BrdU-labeled cells in females that were treated as adults ($F_{1,46} = 1.5, p > 0.05$) or during puberty ($F_{1,26} = 3.16, p > 0.05$).

Experiment 2

Cell numbers were estimated 24 hours after the BrdU injection to determine whether cells undergoing DNA synthesis did in fact divide and produce progeny, rather than exiting the cell cycle and dying (Cameron and McKay, 2001). Omnibus ANOVA indicated that peri-pubescent animals as a group had higher cell numbers than adults ($F_{1,53} = 123.4, p < 0.001$). Peri-pubescent animals produced nearly twice as many new cells (1.8 fold) as adults did during the same period of time ($p = .0001$). There was a significant drug x sex interaction ($F_{1,53} = 6.09, p < 0.05$) but no significant differences using post-hoc analyses. Planned comparisons examining treatment effects within each sex/age group indicated that fluoxetine increased cell proliferation in the hippocampus of adult males ($p = 0.027$) but not in peri-pubescent males ($p = 0.22$), peri-pubescent females ($p = 0.34$) or adult females ($p = 0.52$) (Fig. 3a).

Experiment 3

Cell numbers were estimated 28 days after the BrdU injection to determine whether the newly proliferated cells were incorporated into the granule cell layer differentiated into neurons (Dayer et al., 2003). Omnibus ANOVA indicated a significant interaction between drug treatment and sex ($F_{1,57} = 4.74, p < 0.05$). Post-hoc analysis indicated that females treated with fluoxetine had fewer surviving cells than males treated with fluoxetine ($p = 0.003$). Main effects indicated that 1.6 fold more new cells survived in peri-pubescent rats than adults ($F_{1,57} = 69.89, p < 0.001$) and 1.2 more new cells survived in males than females ($F_{1,57} = 8.85, p < 0.05$). Planned comparisons determined that fluoxetine treatment did not alter cell survival in adult males ($p = 0.27$), peri-pubescent males ($p = 0.29$) or adult females ($p = 0.76$). There was a trend for decreased cell survival in peri-pubescent females ($p = 0.06$) (Fig. 4a).

The phenotype was determined by examining cells that contained both BrdU, a marker of a DNA synthesis and NeuN, a marker of mature neurons. Omnibus ANOVA indicated there were no effects of fluoxetine treatment on cell phenotype ($F_{1,12} = 0.55, p > 0.05$) (Fig. 4b). For all groups, ~ 80% of the BrdU-labeled cells also expressed NeuN as previously reported (Malberg et al., 2000, Encinas et al., 2006). These data indicate that fluoxetine did not alter the proportion of new cells that differentiated into neurons, irrespective of how many cells were generated as a function of age or sex.

Corticosterone levels

Treatment with fluoxetine did not alter corticosterone levels in male and female rats when measured one day after cessation ($F_{1,62} = 0.237, p > 0.05$). Planned comparisons indicated no effect of drug on basal corticosterone concentrations (p values > 0.05 ; Fig. 5a).

To assess the potential long-term impact of fluoxetine on HPA maturation, corticosterone concentrations were assessed 28 days after drug treatment ceased (Fig. 5b). ANOVA indicated a sex $\times$ age $\times$ drug treatment interaction ($F_{1,32} = 4.94, p < 0.05$) and a drug treatment $\times$ age interaction ($F_{1,32} = 7.58, p < 0.05$). Post hoc analysis indicated that females treated with fluoxetine during puberty had lower levels of circulating corticosterone than females treated with saline at the same time period ($p = 0.007$). Planned comparisons of fluoxetine treatment within each age and sex indicated that fluoxetine treatment resulted in a lasting decrease in corticosterone levels for females treated during puberty ($p = 0.003$). Fluoxetine treatment during puberty did not persistently alter corticosterone concentrations in pubescent males ($p = 0.98$) or adults of either sex (males $p = 0.61$ /females $p = 0.35$).

Discussion

Chronic treatment with antidepressants increases the number of new neurons generated in the adult hippocampus. It has been proposed that this increase may explain some of the clinical efficacy of this treatment. As other have, we observed that fluoxetine treatment increased cell proliferation in the hippocampus of the male rat (Malberg et al., 2000, Kodama et al., 2004, Encinas et al., 2006). However, treatment with the same clinically-relevant dose (Alvarez et al., 1998) did not increase cell numbers in females. Recent reports have indicated that higher doses of fluoxetine can increase cell proliferation in female rodents (Airan et al., 2007, Engesser-Cesar et al., 2007, Lagace et al., 2007) and dose response curves should be examined to determine whether there are dose dependent sex differences. Chronic treatment with the tricyclic antidepressant imipramine (15 mg/kg) also has been reported to increase cell proliferation in females (Green and Galea, 2008); therefore, other types of antidepressants should also be examined. Overall, it appears that a higher dose than that typically prescribed for humans is necessary to enhance neurogenesis in females, even accounting for the faster metabolism of rodents (Alvarez et al., 1998, Wyneken et al., 2006).

Chronic fluoxetine administration did not alter neurogenesis in female rats but is still an effective as a treatment for depression in many women. A recent study demonstrated that depressed women were more responsive to SSRI class antidepressants than men (Young et al., 2009). Previously it had been reported that pre-menopausal women were more responsive to SSRI class antidepressants than tricyclic antidepressants (Kornstein et al., 2000, Martenyi et al., 2001). If the data reported in the current study are applicable to humans, then mechanisms other than those related to neurogenesis likely mediate antidepressant efficacy in women. There is some precedence for this as cell proliferation was found to be necessary for antidepressant behavioral efficacy in some strains of male mice (Santarelli et al., 2003) but not others (Holick et al., 2008) using the same tasks, suggesting neurogenesis is not the only mechanism responsible for alleviation of depressive symptomology. It is noted that the current study was conducted in unstressed animals and thus different results could occur in female rats that were stressed or otherwise had some decrease in endogenous neurogenesis. For example, neurogenesis in C57BL/6 mice was increased by fluoxetine treatment only after chronic administration of corticosterone (David et al., 2009).

It is possible that sex differences in the effects of antidepressants on neuroplasticity are mediated by pharmacokinetics but there is a lack of information on this subject in the literature (Yonkers et al., 1992). Plasma levels of fluoxetine were not different between elderly men and women, but women did produce higher levels of the active metabolite norfluoxetine which had slower clearance (Ferguson and Hill, 2006). Norfluoxetine, like fluoxetine is a selective inhibitor of serotonin reuptake (Sanchez and Hyttel, 1999) but has a longer half life in both humans and mice (Alvarez et al., 1998). Increased norfluoxetine content in women may result in longer periods of therapeutic coverage. In addition, norfluoxetine has greater potency than fluoxetine to increase brain content of the neurosteroid allopregnanolone, a positive allosteric

modulator of gamma-aminobutyric acid (Pinna et al., 2006) which has anxiolytic properties when administered systemically. Whether norfluoxetine alters neurogenesis is not known.

The dose of fluoxetine used in the current study did not alter cell proliferation in female rats, although it does affect behaviors related to stress. Daily treatment for 14 days at 5 mg/kg prevented the negative effects of stress on associative learning in female rats (Leuner et al., 2004) demonstrating that this dose is sufficient to ameliorate the effects of stress on a cognitive task which requires the hippocampus. Females are also resistant to manipulations that decrease cell proliferation. For example, stress is known to reduce cell proliferation in males but has minimal to no effect in females (Falconer and Galea, 2003, Airan et al., 2007, Shors et al., 2007). It appears that females may be less sensitive to manipulations that alter cell proliferation resulting in increased ability to maintain homeostasis. However this is only speculation and more research is necessary to determine whether this is in fact the case.

Cells generated during fluoxetine treatment were less likely to survive in females than in males. This effect was largely attributable to the peri-pubescent females; within this group ~20% fewer cells to become neurons in fluoxetine treated animals than saline treated controls. In males, new cells generated during fluoxetine treatment were not incorporated into the granule cell layer in greater numbers than in those treated with saline. These data are in agreement with some (Czeh et al., 2007, Cowen et al., 2008) but not all (Malberg et al., 2000) reports of the effects of fluoxetine on cell survival. Thus, it would appear that fluoxetine caused more new cells to be produced in adult males but did not result in a net increase in neurogenesis. An insult such as stress might alter this response if the new neurons were necessary to restore damage from hippocampal atrophy (Czeh et al., 2007). Also, the cell counts were taken after the drug treatment had ceased. Thus, a continued treatment regime may produce a more lasting influence on neurogenesis, per se.

The current study replicates a previous study showing that this dose increases cell proliferation in males (Malberg et al., 2000, Kodama et al., 2004). However, planned comparisons indicated the increase only occurred in adults suggesting that sexual maturation alters responsiveness of this process to fluoxetine. In mice, sexual maturation also influences the responsiveness of neural plasticity to antidepressant albeit in the opposite direction. Treatment with fluoxetine increased cell proliferation in C57BL/6 mice during puberty but not in adulthood (Navailles et al., 2008).

Peri-pubescent rats of both sexes had increased cell proliferation and survival compared to adults even though there was only approximately a one month difference in their age (Heine et al., 2004, McDonald and Wojtowicz, 2005, Cowen et al., 2008). The present data extend this observation to females. During puberty rats produced 1.8 fold more new cells as their adult counterparts regardless of antidepressant treatment or sex. The increased proliferation lead to increased incorporation of these new cells as peri-pubescent rats had 1.6 fold more new cells than adults 28 days after BrdU labeling regardless of drug treatment and sex. It is possible, given the high rate of proliferation that fluoxetine can not further stimulate cell proliferation beyond some already high point. However other manipulations such as seizure can increase neurogenesis in peri-pubescent rats (Gray et al., 2002) suggesting that the lack of an effect of fluoxetine on cell proliferation is not due to a ceiling effect.

Adult and peri-pubescent rats had different weaning and housing conditions prior to use in the study which could contribute to the age differences reported in the current study. The peri-pubescent animals were weaned between 21–23 days whereas the adults were weaned slightly later at 28 days. Both time points used for weaning were within the standard IACUC guidelines used by animal care facilities. This design was used in order to ensure that all animals were housed singly for 3–5 days before the injections began. It is noted however, that some of the

difference in neurogenesis may be due to differences in the weaning dates. There is a report that very early weaning (PND 14) reduces neurogenesis when compared with the standard age of weaning (PND 21) (Kikusui et al., 2009). Also, adults were group housed, post-weaning and prior to the beginning of the experiment to prevent them from experiencing a longer period of isolation prior to the onset of treatment than the peri-pubescent animals. They were not housed in groups during the experiment because this can lead to changes in proliferation as a result of dominance hierarchies (Kozorovitskiy and Gould, 2004). Since neurogenesis is a dynamic process which is sensitive to so many environmental factors, it is not always possible to keep all conditions the same for all groups. This is especially the case for studies that span transitions from one stage of development to another. Indeed, some of these issues may explain the inconsistent results in the literature with respect to cell proliferation and age effects.

Basal levels of the stress hormone corticosterone were measured because chronic fluoxetine treatment can increase circulating levels of the stress hormone in male mice (Weber et al., 2006). Fluoxetine treatment did not alter circulating levels of corticosterone in rats of either age/sex immediately after the cessation of treatment. Corticosterone levels were examined 28 days after the cessation of fluoxetine treatment because antidepressant treatment during development can alter stress and anxiety related behaviors in adulthood (Ansorge et al., 2004). Fluoxetine treatment of females during puberty altered the basal levels of corticosterone in adulthood. Under normal conditions, basal corticosterone levels increase as females transition from puberty to adulthood and begin their cycle (Hodes and Shors, 2005, Viau et al., 2005). This change did not occur in the females that had been treated chronically with fluoxetine. These data together with the reduced cell survival in females suggests a sex specific vulnerability to fluoxetine treatment during puberty. More research is necessary to determine whether these alterations in HPA axis function and cell survival would have positive or negative influences on emotional experience. A recent report found no effects of adolescent fluoxetine treatment on multiple behavioral measures of fear and anxiety in male mice tested in adulthood (Norcross et al., 2008). Similar studies have not been conducted in females.

Fluoxetine is an effective drug for treating depression in about 60% of humans and is currently the only drug approved by the FDA for treatment of depression in adolescents (FDA). Although suggestive, the data connecting its efficacy with neurogenesis are perhaps limited to depression as it occurs in adulthood and moreover, to that which occurs in men. Minimally, these data indicate that antidepressant drugs such as fluoxetine alter neuronal microstructure and hormonal development differently as a function of age and sex.

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Abbreviations

ANOVA	analysis of variance
BrdU	5-bromo-2'-deoxyuridine
GCL	granule cell layer
HPA axis	

Hypothalamic Pituitary Adrenal axis

NeuN

neuronal nuclear antigen

PND

post natal day

SGZ

subgranular zone

SSRI

Selective serotonin reuptake inhibitor

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Figure 1.

A) Schematic of fluoxetine administration, BrdU labeling, and sacrifice at a 2 hr time point. BrdU labeled cells are labeled in brown and are located in the subgranular zone (SGZ). B) Fluoxetine administration, BrdU labeling and sacrifice at a 24 hr time point. BrdU labeled cells are labeled in brown and appear in clusters located within the SGZ and granule cell layer (GCL). C) Fluoxetine administration, BrdU labeling, and sacrifice 28 days after the cessation of antidepressant treatment. Photomicrographs of peroxidase stained tissue indicate that BrdU labeled cells (in brown) have migrated into the GCL. Photomicrographs of immunofluorescent labeling indicated that many BrdU labeled cells (in red) also displayed a marker for mature neurons (NeuN- in green) suggesting that majority of newly born cells matured into neurons.

Figure 2.

A) Fluoxetine treatment increased cell proliferation at a 2 hr time point when data was averaged together. Peri-pubescent rats had more new cells than adults regardless of drug treatment or sex. Planned comparisons indicated that fluoxetine treatment increased cell proliferation in adult male rats but not male peri-pubescent or female rats at either age. B) Treatment with fluoxetine did not alter cell proliferation at any point of the peri-pubescent estrous cycle. C) Treatment with fluoxetine did not alter cell proliferation at any point in the adult female estrous cycle. Bars represent Mean $\pm$ SEM. Asterisks denote significant ($p < 0.05$) effects of fluoxetine treatment within each sex/age group as determined by planned comparisons.

Figure 3.

A) Cell proliferation was measured 24 hrs after BrdU injection to determine that newly born cells continued through the cell cycle. Fluoxetine treatment increased cell proliferation in male rats as a group compared to females. Peri-pubescent rats had more new cells than adults regardless of drug treatment or sex. Planned comparisons indicated that fluoxetine treatment increased cell proliferation in adult male rats but not peri-pubescent rats or adult females. Bars represent Mean $\pm$ SEM. Asterisks denote significant ($p < 0.05$) effects of fluoxetine treatment within each sex/age group as determined by planned comparisons.

Figure 4.

A) The survival of the newly generated cells was measured by injecting animals with BrdU and waiting 28 days after labeling to sacrifice and stain cells. Females treated with fluoxetine had fewer cells survive than males treated with fluoxetine, there were no sex differences in saline treated animals. More cells survived in peri-pubescent rats than in adults. Planned comparisons indicated that fluoxetine treatment did not alter the number of new cells that survived in adult males, adult females or peri-pubescent males. Fluoxetine treated peri-pubescent females had a trend towards decreased cell survival ($p = 0.06$). B) Newly generated cells that survived for 28 days were double labeled with BrdU and NeuN a marker of mature neurons to determine cell phenotype. Cell fate was not altered by drug treatment and/or sex.

Approximately $79 \pm 9\%$ of the new cells were co-labeled with a neuronal marker. Bars represent Mean $\pm$ SEM. Asterisks denote significant ($p < 0.05$) effects of fluoxetine treatment within each sex/age group as determined by planned comparisons.

Figure 5.

A) Treatment with fluoxetine in peri-pubescent animals did not alter corticosterone levels when measured one day after the cessation of treatment. B) To determine whether fluoxetine treatment had lasting effects on basal levels of corticosterone, hormone levels were examined after a 28 day drug free period. Treatment with fluoxetine during puberty significantly decreased subsequent adult corticosterone levels in females but not males or animals of either sex treated with fluoxetine during adulthood. Bars represent Mean $\pm$ SEM. Asterisks denote significant ($p < 0.05$) effects of fluoxetine treatment within each sex/age group as determined by planned comparisons.