Sex-Specific Effects of Chronic Fluoxetine Treatment on Neuroplasticity and Pharmacokinetics in Mice

Georgia E. Hodes, Tiffany E. Hill-Smith, Raymond F. Suckow, Thomas B. Cooper, and Irwin Lucki

Departments of Psychiatry and Pharmacology, University of Pennsylvania, Philadelphia, Pennsylvania (G.E.H., T.E.H.-S., I.L.); Analytical Psychopharmacology Department, New York State Psychiatric Institute, New York, New York (R.F.S.); and Nathan Klein Institute for Psychiatric Research, Orangeburg, New York (T.B.C.)

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ABSTRACT

Neurogenesis is a mechanism through which antidepressants may produce therapeutic effects. There is a dearth of information regarding the effects of antidepressants on neurogenesis and neurotrophin mobilization in females. This study examined sex differences in the alteration of cell proliferation and survival in multiple regions of the brain. Additional experiments examined brain-derived neurotrophic factor (BDNF) levels and pharmacokinetics of fluoxetine to determine whether they mediate sex differences. MRL/MpJ mice were treated with fluoxetine (5 and 10 mg/kg b.i.d.) for 21 days and received injections of 5-bromo-2'-deoxyuridine (200 mg/kg) to measure DNA synthesis. In the hippocampus, fluoxetine increased cell proliferation at both doses; females treated with 10 mg/kg produced more new cells than males. Fluoxetine did not alter survival in males, but 10 mg/kg reduced survival in females. In the frontal cortex,

fluoxetine increased cell proliferation and survival in males treated with 10 mg/kg. In the cerebellum and amygdala, 10 mg/kg fluoxetine increased cell proliferation in both sexes but did not alter the incorporation of the new cells. Fluoxetine increased BDNF levels in the hippocampus of both sexes. BDNF levels correlated with cell proliferation in males but not females. Brain and plasma levels indicated that females metabolized fluoxetine faster than males and produced more of the metabolite norfluoxetine. These data suggest that fluoxetine acts on multiple areas of the brain to increase cell proliferation, and the pattern of activation differs between males and females. Sex-specific effects of fluoxetine on neurotrophin mobilization and pharmacokinetics may contribute to these differences in neural plasticity.

The neurotrophin/neurogenesis theory of depression proposes that chronic stress associated with depression decreases neurotrophins, leading to decreased neurogenesis. Decreases in neurogenesis and other forms of neural plasticity are thought to lead to impaired hippocampal function (Airan et al., 2007). Antidepressants act to increase the mobilization of neurotrophins and cell proliferation (Duman and Monteggia, 2006). It is postulated that the changes in the mobilization of neurotrophins in turn lead to increased neurogenesis thought to repair damage and restore hippocampal function. A minimum of 2 weeks of continuous treatment with antidepressants is necessary to increase neurogenesis

in the hippocampus because a single acute treatment or continuous treatment up through 7 days does not alter cell proliferation (Balu et al., 2009b; Wu and Castrén, 2009). Long-term treatment with fluoxetine or other antidepressants is also necessary to alter brain-derived neurotrophic factor (BDNF) levels as acute administration does not alter protein or mRNA levels of BDNF in the hippocampus (Nibuya et al., 1995; Balu et al., 2008). Because long-term antidepressant administration is needed to alter cell proliferation and neurotrophin mobilization, it is thought that they might be mechanisms involved in the efficacy of antidepressants to relieve symptoms of depression.

Women are twice as likely as men to experience an episode of depression (Kessler, 2003). This sex difference occurs between puberty (Kaltiala-Heino et al., 2003) and menopause (Sonnenberg et al., 2000), suggesting a biological component to the higher incidence of female depression during reproductive viability. Yet there are relatively few data collected on the effects of antidepressants on neurotrophins and neuro-

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ABBREVIATIONS: BDNF, brain-derived neurotrophic factor; BrdU, 5-bromo-2-deoxyuridine; 7-AAD, 7-aminoactinomycin D; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.

genesis in females. To date, two studies done on females indicated that a high dose of fluoxetine (20–25 mg/kg) increased neurogenesis in the hippocampus of female mice (Engesser-Cesar et al., 2007) and rats (Airan et al., 2007) after multiple injections of 5-bromo-2-deoxyuridine (BrdU). The only study that directly compared the effects of fluoxetine on cell proliferation and survival between the sexes did not examine the dose-response relationship (Lagace et al., 2007).

The current study directly compares the effects of long-term administration of fluoxetine on cell proliferation and survival in multiple regions of the brain of male and female mice. It has been accepted that neurogenesis occurs in the dentate gyrus of the hippocampus and the subventricular zone. There has been controversy as to whether neurogenesis also occurs in other areas of the brain, such as the frontal cortex (Kodama et al., 2004; Dayer et al., 2005; Czéh et al., 2007; Cameron and Dayer, 2008). It has been difficult to measure cell genesis in these areas with immunohistochemistry because neural progenitors are sparse and distributed diffusely (Cameron and Dayer, 2008). Recent reports suggest that antidepressant treatments can alter cell proliferation and neurogenesis in other areas of the brain. In vitro treatment of cells taken from the cerebellum with fluoxetine caused a 50% increase in the number of cells that matured into neurons (Zusso et al., 2008). In the amygdala, electroconvulsive shock is capable of increasing proliferation in rats (Wennström et al., 2004). In the present study, the effects of long-term fluoxetine administration on cell proliferation and survival were examined in the hippocampus, subventricular zone, frontal cortex, amygdala, and cerebellum. This was enabled by the use of flow cytometry, which can measure the incorporation of BrdU rapidly in various tissues. The effects of long-term fluoxetine treatment on the neurotrophin BDNF were also examined in the same animals to determine whether there was a relationship between neurotrophin mobilization and neurogenesis in the hippocampus. Finally, steady-state levels of fluoxetine and its metabolite norfluoxetine were examined to determine whether pharmacokinetics and metabolism mediated sex differences in neural plasticity.

Materials and Methods

Animals. Adult male and female MRL/MpJ mice (The Jackson Laboratory, Bar Harbor, ME) were 7 to 10 weeks old at the beginning of all the studies. MRL/MpJ mice show enhanced regenerative responses to injury, and males show robust increases in neurogenesis after long-term treatment with both fluoxetine and desipramine (Balu et al., 2009a). Mice were group-housed (five to a cage, single sex) in polycarbonate cages and maintained on a 12-h light/dark cycle (lights on at 7:00 AM) in a temperature (22°C) and humidity-controlled colony. Animals were given ad libitum access to food and water. All the procedures were conducted in accordance with the guidelines published in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996), and all the protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Female mice were tracked for the estrous cycle via visual inspection during daily injections. During the stage of estrus MRL/MpJ females displayed a visible discharge of cells, and the occurrence of this discharge was recorded every 4 to 5 days to determine whether females were cycling. Vaginal swabs were only taken after sacrifice as they are reported to decrease cell proliferation in mice (Lagace et

al., 2007). For the proliferation study, 26 of 30 mice were in estrus at time of sacrifice. For the survival study, 23 of 30 mice were in estrus at the time of sacrifice.

Drugs. All the drugs were given by intraperitoneal injection. Fluoxetine hydrochloride was obtained from Anawa (Zurich, Switzerland) and was prepared fresh daily. The doses were calculated according to the base weight of the drug and administered in a volume of 10 ml/kg. BrdU (Roche Applied Science, Indianapolis, IN) was dissolved in warm saline and administered by intraperitoneal injection at a volume of 10 ml/kg.

Injections. Mice were administered injections of 0.9% saline or fluoxetine (5 or 10 mg/kg) twice daily for 21 days ($n = 6$ –10/group). For the cell proliferation study, animals ($n = 60$; 10 per dose/sex) received antidepressant treatment for 21 days. On the last day of treatment, mice received a single injection of BrdU (200 mg/kg) and were sacrificed 24 h later. To measure the number of new cells that survived and were incorporated, mice were given injections of fluoxetine for 21 days ($n = 60$; 10 per dose/sex), then received a single injection of BrdU (200 mg/kg) at the end of treatment, and were sacrificed 28 days later. For the proliferation study, two males in the 10 mg/kg treatment group died before sacrifice. For the survival study, three males in the 10 mg/kg treatment group died before sacrifice.

BrdU Incorporation Using Flow Cytometry. Labeling of BrdU was measured in cells displaying the nuclear marker 7-aminoactinomycin D (7-AAD) by flow cytometry as previously described and validated (Balu et al., 2009a,b). Mice were decapitated and their brains were quickly removed and dissected into the following regions: hippocampus, frontal cortex, subventricular zone, amygdala, and cerebellum. For the hippocampus, only the right lobe was analyzed, as cell counts did not differ between hemispheres (Balu et al., 2009a). All the other brain regions were dissected and combined bilaterally. All the brain areas were placed in Hanks' balanced salt solution (Invitrogen, Carlsbad, CA) and finely minced. Tissue was digested using an enzymatic mixture (0.5 ml, 1 mg/ml papain; Roche Applied Science; 0.1 M L-cysteine; Sigma-Aldrich, St. Louis, MO) and incubated in a dry heat block at 37°C for 15 min. Hibernat-A (Brain Bits LCC, Springfield, IL) containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) was added to stop the enzymatic digestion. Tissue was then mechanically triturated to form a single-cell suspension and spun in a centrifuge at 2000 rpm for 5 min.

The supernatant was removed, and the resultant cells were stained using the fluorescein isothiocyanate (FITC) BrdU Flow Kit (BD Biosciences, San Jose, CA). Cells were initially fixed and permeabilized by resuspension in 100 μ l of Cytofix/Cytoperm buffer (BD Biosciences). Cells were then washed in staining buffer [phosphate-buffered saline (PBS), 3% FBS, 0.09% sodium azide], spun at 5000 rpm, and aspirated. To store cells until they could be analyzed with cohorts, 500 μ l of freezing medium (10% dimethyl sulfoxide, 90% FBS) was added, and cells were then stored at -80°C . When all of the samples within an anatomical area had been collected, cells were thawed in a water bath (37°C, 1.5 min) and washed in staining buffer. Cells were then refixed (5 min) and washed. The cells were then resuspended in 100 μ l of DNase [30 μ g; stock from kit was diluted in Dulbecco's PBS ($\text{Ca}^{2+}/\text{Mg}^{2+}$ -free) containing 0.1 mM CaCl_2 and 10 mM MgCl_2] in a dry heat block at 37°C for 1 h to break down DNA into a single strand and allow bonding of the BrdU antibody. After washing and spinning, the cells were labeled with 50 μ l of FITC-conjugated anti-BrdU (1:50 dilution) in the dark at room temperature for 20 min. After the samples were washed, they were labeled with 20 μ l of the nuclear marker, 7-AAD, at room temperature in the dark. The cells were then resuspended in staining buffer (PBS, 3% FBS, 0.09% sodium azide). Before analysis, cells were filtered through a cell strainer cap (30 μ m) to remove debris. The data were collected on a BD FACS Canto system (BD Biosciences) at the University of Pennsylvania Flow Cytometry Core Facility. Background signal was controlled for by staining tissue from animals that

had not received injections of BrdU. All the data were collected and analyzed using BD FACSDiva software (BD Biosciences).

BDNF Protein. The left lobe of the hippocampus was used to measure BDNF levels 24 h after cessation of antidepressant treatment (proliferation study) and 28 days after cessation of antidepressant treatment (survival study). Tissue was flash-frozen in isopentane and stored at -80°C until analysis. BDNF protein levels were quantified using a commercially available sandwich enzyme-linked immunosorbent assay kit (Promega, Madison, WI). The tissue was homogenized in 0.75 ml of lysis buffer (100 mM PIPES, pH 7.0, 500 mM NaCl, 2 mM EDTA, 0.1% sodium azide, 2% bovine serum albumin, 0.2% Triton X-100, 5 $\mu\text{g}/\text{ml}$ aprotinin, 0.1 $\mu\text{g}/\text{ml}$ pepstatin A, and 0.5 $\mu\text{g}/\text{ml}$ antipain). The homogenate was centrifuged at 2500 rpm for 30 min at 4°C . The supernatant was removed, and the amount of BDNF protein in each sample was measured in duplicate by enzyme-linked immunosorbent assay. BDNF levels were normalized to the wet tissue weight.

Fluoxetine Levels. A separate group of male ($n = 14$) and female mice ($n = 14$) was treated with 5 or 10 mg/kg i.p. fluoxetine (b.i.d.) for 5 days to achieve a steady state (Holladay et al., 1998). On the 6th day of treatment mice were sacrificed 2 h after the morning injection. Brains were removed and flash-frozen in isopentane and stored frozen (-80°C) until analysis. Trunk blood was removed at sacrifice and placed in EDTA-treated capillary tubes (Sarstedt, Nümbrecht, Germany). Tubes were spun in a centrifuge at 3000 rpm for 15 min. Plasma aliquots were stored frozen (-80°C) until analysis.

Plasma and brain fluoxetine and its major metabolite norfluoxetine were determined by high-pressure liquid chromatography with fluorescence detection as described previously (Suckow et al., 1992). In brief, plasma (0.1 ml) or brain homogenate samples (0.5 ml) were rendered alkaline and extracted with 20% ethyl acetate in *n*-heptane. After evaporation of the solvent extract, the residue was derivatized with dansyl chloride. The highly fluorescent derivatives were chromatographed on a reversed-phase C-18 column with a mobile phase of phosphate buffer and acetonitrile. Total run time, including the internal standard maprotiline, was less than 14 min with no interference from endogenous material. The six-point calibration curve was linear over the concentration range 25 to 800 ng/ml with inter- and intra-assay imprecision (CV) of $<10\%$ ($n = 11$ for each concentration). The lower limit of quantitation was 3 ng/ml for both fluoxetine and norfluoxetine, with an intra-assay variation of 9.8 and 4.9% ($n = 11$ for each), respectively.

Statistical Analysis. Statistical analysis was performed using analysis of variance, and post hoc analysis was performed using Newman-Keuls. All the analyses were performed as two-way analysis of variance (drug dose $\times$ sex) using Statistica software (StatSoft, Tulsa, OK).

Results

Effects of Fluoxetine Treatment on Cell Proliferation and Survival in Multiple Brain Regions. Cell proliferation was measured 24 h after BrdU injection to determine whether fluoxetine injections for 21 days produced sex-specific alterations in multiple regions of the brain. Cell counts were examined in a separate set of animals 28 days after BrdU labeling to determine whether the new cells created during the course of fluoxetine treatment survived and were incorporated into brain regions.

In the hippocampus, a significant interaction between sex and dose indicated that male and female mice displayed different patterns of cell proliferation in response to treatment with fluoxetine ($F_{2,50} = 6.32$; $p < 0.01$). The dose-response curve in males had an inverted U-shaped function; the 5-mg/kg dose of fluoxetine produced the largest increase in cell proliferation ($p < 0.05$; Fig. 1A). For females, the

Fig. 1. Twenty-one days of intraperitoneal injections (b.i.d.) with 5 or 10 mg/kg fluoxetine (FLX) produced different dose-dependent patterns of cell proliferation ($n = 7$ –10 per dose/sex) and survival ($n = 7$ –10 per dose/sex) in the hippocampus of male and female MRL/MpJ mice. A, treatment with FLX significantly increased cell proliferation in male mice compared with saline-injected controls. Males treated with 5 mg/kg produced more new cells than those treated with 10 mg/kg. B, treatment with FLX significantly increased cell proliferation in the hippocampus of female mice compared with saline-treated controls. Females treated with 10 mg/kg FLX had higher levels of cell proliferation than their male counterparts and females treated with 5 mg/kg. C, long-term FLX treatment with 5 or 10 mg/kg did not alter cell survival in the hippocampus of male mice compared with saline-injected controls. D, long-term FLX treatment decreased cell survival in the hippocampus of female mice treated with 10 mg/kg FLX compared with females that received injections of 5 mg/kg, males that received injections of 10 mg/kg, and saline-treated controls. Bars represent mean values $\pm$ S.E.M. Asterisk (*) denotes significant differences within sex, and daggers (^) denote differences between the sexes. Multiple asterisks/daggers indicate level of significance (*, $p < 0.05$; **, $p < 0.01$).

dose-response curve displayed a step-wise function. Higher doses of fluoxetine resulted in significantly higher levels of cell proliferation ($p < 0.05$; Fig. 1B). Post hoc analysis with Newman-Keuls indicated that 5 mg/kg fluoxetine increased cell proliferation to equal levels in both sexes. At the highest dose (10 mg/kg) females produced almost twice the number of new cells as males ($p = 0.003$). Fluoxetine also had different dose-dependent effects on cell survival in males and females as indicated by a significant interaction ($F_{2,47} = 4.34$; $p < 0.05$). In females but not males there was a 2-fold reduction of cell survival in animals treated with the highest dose of fluoxetine (10 mg/kg). Females treated with the high dose of fluoxetine had significantly fewer BrdU-labeled cells than male and female saline controls, females treated with the lower dose (5 mg/kg), and males treated with 10 mg/kg ($p < 0.05$) (Fig. 1D). In males fluoxetine produced no alteration in cell survival (Fig. 1C).

In the subventricular zone, fluoxetine treatment did not alter cell proliferation at either dose tested ($F_{2,45} = 0.52$; $p > 0.05$). There were no sex differences ($F_{1,45} = 1.93$; $p > 0.05$) and no interactions between sex and treatment ($F_{2,45} = 0.17$;

$p > 0.05$) (data not shown). Cell survival was not examined in this region.

Fluoxetine treatment increased cell proliferation of males but not females in the frontal cortex, as indicated by a significant interaction between treatment and sex ($F_{2,46} = 4.50$; $p < 0.05$). There was a 3-fold increase in cell proliferation in the frontal cortex for male mice treated with the highest dose of fluoxetine (10 mg/kg) compared with controls ($p = 0.007$) (Fig. 2A), whereas fluoxetine treatment did not alter cell proliferation in female mice or males treated with the lower dose ($p > 0.05$) (Fig. 2B). Long-term fluoxetine treatment (10 mg/kg) resulted in sex-specific alterations in the number of surviving new cells in the frontal cortex as indicated by an interaction between sex and treatment ($F_{2,48} = 12.23$; $p < 0.001$). In males, the lower dose (5 mg/kg) of fluoxetine resulted in a 1.8-fold decrease cell survival compared with same sex saline controls ($p = 0.03$), whereas the high dose (10 mg/kg) resulted in a 1.6-fold increase in cell survival ($p = 0.0003$) (Fig. 2C). Fluoxetine treatment did not alter cell survival in females. Saline-treated males had 1.6-fold more surviving BrdU-labeled cells in the frontal cortex compared with females (Fig. 2D).

In the cerebellum, fluoxetine treatment increased cell proliferation in both males and females as indicated by a main

Fig. 2. Twenty-one days of intraperitoneal injections (b.i.d.) with 5 or 10 mg/kg FLX produced different dose-dependent patterns of cell proliferation ($n = 7$ –10 per dose/sex) and survival ($n = 6$ –10 per dose/sex) in the frontal cortex of male and female MRL/MpJ mice. A, treatment of male mice with 10 mg/kg but not 5 mg/kg FLX significantly increased cell proliferation compared with saline-injected controls and females of all the treatments. B, long-term treatment with FLX did not alter cell proliferation in the frontal cortex of females. C, long-term treatment with FLX altered the survival of new cells in the frontal cortex of male mice. Treatment with 5 mg/kg FLX reduced cell survival, whereas treatment with 10 mg/kg increased cell survival compared with saline-injected controls. D, FLX treatment did not alter cell survival in the frontal cortex of female mice. Fewer new cells survived in females than in saline-injected males. Bars represent mean values $\pm$ S.E.M. Asterisk (*) denotes significant differences within sex, and daggers (^) denote differences between the sexes. Multiple asterisks/daggers indicate level of significance (*, $p < 0.05$; **, $p < 0.01$).

effect of treatment ($F_{2,51} = 3.66$; $p < 0.05$) (Fig. 3, A and B). Post hoc analysis determined that the highest dose (10 mg/kg) resulted in a 1.5-fold increase in cell proliferation for the cerebellum when data were collapsed across sex ($p = 0.027$). Females had higher levels of cell proliferation regardless of drug treatment ($F_{1,51} = 10.62$; $p < 0.01$). Females produced on average 1.5 cells for every cell males produced in the cerebellum. There were no interactions between sex and drug treatment ($F_{2,51} = 0.05$; $p > 0.05$). Treatment with fluoxetine did not alter cell survival in the cerebellum of mice regardless of sex ($F_{2,49} = 0.86$; $p > 0.05$). However, cell survival was reduced in females compared with males in data averaged across treatment groups ($F_{1,49} = 11.83$; $p < 0.01$) (Fig. 3, C and D). There was a 2-fold decrease in the survival of new cells in females compared with males. There were no interactions between sex and treatment ($F_{2,49} = 2.19$; $p > 0.05$).

Long-term treatment with fluoxetine increased cell proliferation in the amygdala of male and female mice ($F_{2,46} = 14.45$; $p < 0.001$) when data were collapsed across sex (Fig. 4, A and B). There was a 3-fold increase in the number of new cells compared with saline-treated controls or animals given the lower dose of fluoxetine (5 mg/kg) when data were averaged between the sexes ($p = 0.0001$). Examination of the means within sexes determined that males produced approx-

Fig. 3. Effects of 21 days of intraperitoneal injections of FLX (5 and 10 mg/kg b.i.d.) on cell proliferation ($n = 8$ –10 per dose/sex) and survival ($n = 6$ –10 per dose/sex) in the cerebellum of the male and female MRL/MpJ mouse. A, 10 mg/kg but not 5 mg/kg increased cell proliferation in the cerebellum of male mice compared with saline-injected controls when data were collapsed across sex. B, 10 mg/kg but not 5 mg/kg FLX increased cell proliferation above levels produced in saline-injected female mice when data were collapsed across sex. Females had higher levels of cell proliferation than males regardless of drug treatment. C, FLX treatment did not alter cell survival in the cerebellum in male mice. D, FLX treatment did not alter cell survival in females at any dose. Fewer new cells survived in females than in males regardless of treatment. Bars represent mean values $\pm$ S.E.M. Asterisk (*) denotes significant differences within sex, and daggers (^) denote differences between the sexes. Multiple asterisks/daggers indicate level of significance (*, $p < 0.05$; **, $p < 0.01$).

Fig. 4. Effects of 21 days of intraperitoneal injections of FLX (5 and 10 mg/kg b.i.d.) on cell proliferation ($n = 7-10$ per dose/sex) and survival ($n = 6-10$ per dose/sex) in the amygdala of the male and female MRL/MpJ mouse. A, 10 mg/kg increased cell proliferation in the amygdala of male mice compared with 5 mg/kg FLX and saline-treated controls when data were collapsed across sex. B, for females, 10 mg/kg FLX increased cell proliferation saline-treated controls when data were collapsed across sex. C, FLX treatment did not alter cell survival in the amygdala in male mice. D, FLX treatment did not alter cell survival in females at any dose. Bars represent mean values $\pm$ S.E.M. Asterisk (*) denotes significant differences within sex, and daggers (†) denote differences between the sexes. Multiple asterisks/daggers indicate level of significance (*, $p < 0.05$; **, $p < 0.01$).

imately four cells for every new cell produced by same sex controls, whereas females only produced a 2-fold increase in proliferation compared with their same sex cohorts. There was no significant sex difference in the number of proliferating cells in the amygdala ($F_{1,46} = 1.98$; $p > 0.05$) and no interactions between drug treatment and sex on cell proliferation ($F_{2,46} = 2.04$; $p > 0.05$). Fluoxetine treatment did not alter cell survival in the amygdala ($F_{2,47} = 1.21$; $p > 0.05$) (Fig. 4, C and D). There were no sex differences in the survival of new cells in the amygdala ($F_{1,47} = 0.09$; $p > 0.05$) and no interactions between treatment and sex ($F_{2,47} = 0.55$; $p > 0.05$).

Effects of Fluoxetine Treatment on BDNF Levels. Levels of BDNF in the hippocampus were examined in male and female mice 1 day after the cessation of long-term treatment with fluoxetine to determine whether changes in protein levels mirrored changes in cell proliferation. Long-term treatment with fluoxetine increased BDNF levels in the hippocampus of male and female mice ($F_{2,50} = 6.09$; $p < 0.01$) (Fig. 5, A and B). Treatment with either dose of fluoxetine caused a 1.5-fold increase in BDNF levels in the hippocampus. There was no sex difference ($F_{1,50} = 1.08$; $p > 0.05$) and no interaction between treatment and sex ($F_{2,50} = 1.12$; $p > 0.05$). BDNF levels correlated with cell proliferation measured in the contralateral hippocampus of male ($r = 0.41$; $p < 0.05$) (Fig. 5C) but not female mice ($r = 0.05$; $p > 0.05$) (Fig. 5D).

Fig. 5. Effects of 21 days of intraperitoneal injections with FLX (5 and 10 mg/kg b.i.d.) on BDNF levels in the hippocampus measured 24 h ($n = 8-10$ per dose/sex) and 28 days ($n = 7-10$ per dose/sex) after the cessation of treatment. A, long-term treatment with both doses of FLX increased BDNF levels in males compared with saline-injected controls when data were collapsed across sex. B, both doses of FLX increased BDNF levels in females compared with saline controls when data were collapsed across sex. C, levels of BDNF measured in the hippocampus of male animals significantly correlated with cell proliferation in the contralateral hippocampus. D, levels of BDNF measured in the hippocampus of female mice did not correlate with cell proliferation in the contralateral hippocampus. E, BDNF levels were no different from saline-treated controls 28 days after cessation of treatment in males. F, the effects of FLX treatment on BDNF levels were also not long-lasting in females. Bars represent mean values $\pm$ S.E.M. Asterisk (*) denotes significant differences within sex, and daggers (†) denote differences between the sexes. Multiple asterisks/daggers indicate level of significance (*, $p < 0.05$; **, $p < 0.01$).

BDNF levels in the hippocampus were examined 28 days after the cessation of fluoxetine treatment to determine whether changes in neurotrophin mobilization were long-lasting (Fig. 5, E and F). There were no lasting effects of previous fluoxetine treatment on BDNF levels in mice ($F_{2,51} = 0.515$; $p > 0.05$). Males and females did not differ significantly in the amount of BDNF ($F_{1,51} = 1.94$; $p > 0.05$), and there was no significant interaction between previous treatment and sex ($F_{2,51} = 2.60$; $p > 0.05$).

Fluoxetine Levels in Brain and Plasma. Fluoxetine and norfluoxetine levels in the brain and plasma were measured to ascertain sex differences in the pharmacokinetics of the antidepressant after a steady state had been achieved. Levels of fluoxetine and its active metabolite norfluoxetine in the brain and plasma are presented in Table 1. As expected,

TABLE 1

Circulating levels of fluoxetine and norfluoxetine

Display of the circulating levels of fluoxetine and its metabolite norfluoxetine (mean $\pm$ S.E.M.) in the brain and plasma of male and female mice. Significant sex difference indicated by ^a within column. Significant effect of dose indicated by ^b within row. Fluoxetine and norfluoxetine levels in the brain and plasma were measured after injections (5 and 10 mg/kg b.i.d.) with fluoxetine for 6 days to achieve a steady state. Mice were sacrificed 2 h after the morning injection on day 6.

	5 mg/kg		10 mg/kg	
	Fluoxetine	Norfluoxetine	Fluoxetine	Norfluoxetine
Brain (μ g/g)				
Male	16 $\pm$ 0.5	10 $\pm$ 0.3	39 $\pm$ 3.6 ^b	19 $\pm$ 1.3 ^b
Female	11 $\pm$ 0.9	9 $\pm$ 0.8	36 $\pm$ 1.2 ^b	22 $\pm$ 0.9 ^{a,b}
Plasma (ng/ml)				
Male	817 $\pm$ 34	800 $\pm$ 41	1956 $\pm$ 116 ^{a,b}	1426 $\pm$ 40 ^b
Female	641 $\pm$ 23	894 $\pm$ 39	1715 $\pm$ 52 ^b	1607 $\pm$ 55 ^{a,b}

10 mg/kg fluoxetine resulted in higher levels of fluoxetine in the brain ($F_{1,24} = 144.31$; $p < 0.001$) and the plasma ($F_{1,23} = 257.45$; $p < 0.001$) than 5 mg/kg. Males had higher levels of fluoxetine than females in the brain ($F_{1,24} = 4.55$; $p < 0.05$) and the plasma ($F_{1,23} = 9.14$; $p < 0.01$). There were no interactions between dose and sex in the brain ($F_{1,24} = 0.42$; $p > 0.05$) or the plasma ($F_{1,23} = 0.23$; $p > 0.05$). Analysis of brain levels of norfluoxetine resulted in an interaction between dose and sex ($F_{1,24} = 4.24$; $p < 0.05$). At the low dose (5 mg/kg) males and females did not differ in the amount of norfluoxetine present in the whole brain, whereas at the high dose (10 mg/kg) females had higher brain levels of norfluoxetine than males ($p = 0.01$). In the plasma, there was no interaction between dose and sex on levels of norfluoxetine ($F_{1,23} = 0.96$; $p > 0.05$). In the plasma 10 mg/kg fluoxetine resulted in higher levels of norfluoxetine ($F_{1,23} = 276.6$; $p < 0.001$) than 5 mg/kg. Females had higher plasma levels of norfluoxetine than males ($F_{1,23} = 9.6$; $p < 0.05$).

To examine the metabolism of the drug and understand its relationship to its metabolite, norfluoxetine to fluoxetine ratios were calculated and analyzed for males and females at both doses (5 and 10 mg/kg) (Fig. 6A). There was a significant

interaction between dose and sex ($F_{1,24} = 9.6$; $p < 0.01$) for whole brain levels of metabolite to parent. At the lower dose (5 mg/kg) females produced more norfluoxetine from fluoxetine, displaying approximately a 1.5-fold increase in the ratio of metabolite to parent. At the higher dose (10 mg/kg) females also had higher levels of metabolite to parent than males but to a lesser degree (1.25-fold difference). Both sexes had lower ratios of metabolite to parent than their same sex cohorts when they were treated with 10 mg/kg, probably indicating saturation in the metabolism of fluoxetine.

Analysis of ratios of metabolite to parent in the plasma also resulted in a significant interaction between dose and sex ($F_{1,23} = 16.32$; $p < 0.001$) (Fig. 6B). At the lower dose females had approximately 1.5-fold increase in the level of norfluoxetine to fluoxetine over males, whereas at higher doses the difference was smaller (1.25-fold difference). Both sexes produced lower ratios of metabolite to parent at the higher dose.

Discussion

This study shows for the first time that fluoxetine induces dose-dependent sex differences on cell proliferation and survival in multiple regions of the brain. Mobilization of the neurotrophin BDNF and the pharmacokinetic activity of fluoxetine were examined as possible mechanisms.

Effects of Fluoxetine Treatment on Cell Proliferation and Survival. Between 80 and 90% of BrdU-labeled cells in the hippocampus develop a neuronal phenotype 28 days after labeling (Malberg et al., 2000; Cameron and McKay, 2001; Encinas et al., 2006), suggesting that the majority of new cells measured in this region would become neurons. Male and female MRL/MpJ mice produced different dose-dependent patterns of cell proliferation in the hippocampus after long-term treatment with fluoxetine. In males, the low dose of fluoxetine produced the largest increase in proliferation, whereas the high dose produced more new cells in females. Cells born during treatment with 10 mg/kg fluoxetine but not 5 mg/kg had a reduced probability of survival in females. Fluoxetine treatment did not alter survival in males. Other studies report increased incorporation of new neurons in the hippocampus produced during fluoxetine treatment (Malberg et al., 2000; Encinas et al., 2006), and discrepancies may stem from strain or species differences.

Neurogenesis occurs in the subventricular zone. Neuroblasts are born in the subventricular zone and then migrate along the rostral migratory stream to the olfactory bulb where they mature into interneurons (Lois et al., 1996). Treatment with fluoxetine does not alter cell proliferation in the subventricular zone of male rats (Malberg et al., 2000). The present study replicated these earlier results in male rats and extended them to male and female mice.

The existence of neurogenesis in the frontal cortex of rodents and primates is controversial (Cameron and Dayer, 2008). Treatment with 10 mg/kg fluoxetine increased cell proliferation and survival in males. Fluoxetine treatment did not alter proliferation or survival in females but had lower levels of survival in the frontal cortex than males. Treatment with the lower dose of fluoxetine in males decreased cell survival compared with saline-treated controls. It is currently understood that dividing cells in the frontal cortex express NG2, an immature oligodendrocyte marker (Bela-

Fig. 6. The ratio of norfluoxetine (NFLX) to FLX in the brains and plasma of male and female MRL/MpJ mice treated for 5 days with 5 and 10 mg/kg FLX (b.i.d.) to achieve a steady state ($n = 6-7$ per dose/sex). A, females produced more NFLX from FLX than males at both doses when levels were measured from the whole brain. Mice treated with 10 mg/kg had a smaller ratio of parent to metabolite than same sex cohorts treated with 5 mg/kg, suggesting saturation of the brain occurs with the higher dose. B, females produced more NFLX from FLX than males at both doses when circulating levels were measured in the plasma. Mice treated with 10 mg/kg had a smaller ratio of parent to metabolite than same sex cohorts treated with 5 mg/kg, suggesting saturation of the blood occurs with the higher dose. Bars represent mean values $\pm$ S.E.M. Asterisk (*) denotes significant differences within sex, and daggers (†) denote differences between the sexes. Multiple asterisks/daggers indicate level of significance (*, $p < 0.05$; **, $p < 0.01$).

chew et al., 2003; Dayer et al., 2005; Czéh et al., 2007). There is evidence that a subpopulation of these cells can also be colabeled with neuronal markers (Dayer et al., 2005) and have electrophysiological properties of immature neurons (Belachew et al., 2003).

Recent articles have suggested that neural stem cells in the adult mouse cerebellum (Klein et al., 2005; Sottile et al., 2006) may also be capable of generating granule cell neurons. Long-term treatment with 10 mg/kg fluoxetine increased cell proliferation in the cerebellum of male and female mice. Female mice produced more new cells than males, although fewer of them were incorporated into the cerebellum. Fluoxetine treatment did not alter cell survival in either sex. Treatment of cells taken from the cerebellum of young rats with fluoxetine *in vitro* has been shown to increase the number of proliferating cells resulting in a larger number of new cells with a neuronal phenotype (Zusso et al., 2008).

There is evidence that neurogenesis may occur in the amygdala of rats (Keilhoff et al., 2006). Treatment with the higher dose of fluoxetine increased cell proliferation of both sexes in the amygdala but did not alter cell survival in either sex. This was the only region in which there were no significant gender differences. In rats, olfactory bulbectomy increased the number of new cells in the basolateral amygdala, and approximately 85% of them expressed a neuronal marker (Keilhoff et al., 2006). Treatment with imipramine did not alter the number of new cells in the amygdala or prevent the effects of bulbectomy on cell genesis in this region. In addition, electroconvulsive shock has been reported to increase newborn cells in the amygdala, and because they displayed the marker for NG2, they were thought to mature into glia (Wennström et al., 2004).

The present study indicates that fluoxetine treatment induces sex differences in the pattern of cell proliferation and survival across the brain. Male mice produced the largest increase in proliferation in the hippocampus at a dose that decreased cell survival in the frontal cortex and caused no other changes in the examined regions. Females were most responsive in the hippocampus to a higher dose, which also increased cell proliferation in the cerebellum and the amygdala, suggesting that plasticity in multiple regions of the brain may be involved in antidepressant efficacy in females. Females overall displayed decreased cell survival compared with males in every region except the amygdala, an extension of research reported from the rat hippocampus (Galea, 2008).

These sex differences may be mediated, in part, by gonadal hormones. Fluoxetine treatment may have reduced estrogen levels resulting in a permanent estrus because the majority of females were in estrus at the time of sacrifice. Visual inspection of discharge suggested that females continued to cycle during the treatment; however, fluoxetine treatment can disrupt cycling in the female rat depending on strain (Maswood et al., 2008), and little is known about how it affects the cycle of female mice. In the rat, the stages of the estrous cycle did not alter the effects of fluoxetine on neurogenesis when females were treated with 5 mg/kg (Hodes et al., 2009), but this may be species/strain-specific. Certainly both male and female gonadal hormones can alter neurogenesis in the hippocampus and could contribute to the reported sex differences. In females, gonadal hormones generally alter proliferation more than survival, whereas in

males gonadal hormones seem to alter survival more than proliferation (Galea, 2008).

Neurotrophin Mobilization. Antidepressants may act to increase neurogenesis by increasing the mobilization of neurotrophins (Duman and Monteggia, 2006). Long-term treatment with fluoxetine at both doses increased BDNF expression in male and female mice. However, BDNF levels correlated with hippocampal cell proliferation in males but not females. These data support BDNF as a mechanism through which fluoxetine can drive increased hippocampal cell proliferation, but this mechanism may be more operational in males than in females. In male MRL/MpJ mice, BDNF levels were also elevated after fluoxetine treatment with 10 mg/kg but not 5 mg/kg in the frontal cortex and the amygdala (Balu et al., 2009a), regions in which this report found corresponding increases in cell proliferation. Future research on BDNF levels in these regions in females along with studies of sex differences in TrkB receptor affinity and expression are needed to fully understand the relationship between BDNF mobilization and cell proliferation.

Once antidepressant treatment ceased, BDNF protein expression returned to pretreatment levels, indicating that the effects of antidepressant treatment were not long-lasting. In females decreased cell survival in the hippocampus was not mirrored by decreases in BDNF levels.

Sex Differences in the Pharmacokinetics of Fluoxetine. Circulating levels of fluoxetine and norfluoxetine in the brain and plasma were measured after steady state had been achieved to determine whether there were pharmacokinetic differences between sexes. Female mice were more efficient at metabolizing fluoxetine than males. Females produced lower levels of fluoxetine, higher levels of its major metabolite norfluoxetine, and maintained a higher metabolite/parent ratio in both plasma and brain. There is little published information on sex differences in the metabolism of fluoxetine in humans. In one study, elderly males and females did not differ in plasma concentrations of fluoxetine, but women had significantly higher levels of norfluoxetine than men and slower clearance of norfluoxetine (Ferguson and Hill, 2006). This pattern is similar to the one measured here in mice and suggests that activational reproductive hormones are not necessary for sex differences in fluoxetine metabolism. Under clinical treatment, women may also receive higher actual doses of fluoxetine when the fixed doses ordinarily prescribed are normalized for body weight. The cytochrome P450 family of enzymes is involved in the metabolism of fluoxetine (Preskorn, 1997; Anderson, 2005) and includes CYP2D6, CYP2C19, and CYP3A4. CYP3A4 has approximately 50% increased enzymatic activity in human females (Anderson, 2005) and may contribute to the increased metabolism of fluoxetine.

Sex differences in the pharmacokinetics of fluoxetine may act to alter plasticity and antidepressant efficacy through both serotonin reuptake-dependent and -independent pathways. Like fluoxetine, norfluoxetine is a selective inhibitor of serotonin reuptake (Sánchez and Hyttel, 1999), but its longer half-life in both humans and mice (Alvarez et al., 1998) may translate to longer periods of clinically therapeutic coverage in females. Norfluoxetine has greater potency than fluoxetine to increase the brain content of the neurosteroid allopregnanolone, an allosteric modulator of GABA (Pinna et al., 2006). Allopregnanolone increased the proliferation of neu-

roprogenitor cells in rat and human tissue (Wang et al., 2005). In addition, fluoxetine is a more potent antagonist of the 5-HT_{2C} receptor than norfluoxetine (Pälvimäki et al., 1996), and sustained antagonism of the 5-HT_{2A/2C} receptors can increase cell proliferation in the hippocampus (Jha et al., 2008). Thus, pharmacokinetic differences in fluoxetine metabolism may contribute to sex differences in the effects of fluoxetine on neurogenesis, causing females to respond to higher doses of fluoxetine than males with greater brain plasticity.

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Address correspondence to: Irwin Lucki, University of Pennsylvania, Departments of Psychiatry and Pharmacology, Translational Research Building, 125 South 31st Street, Room 2204, Philadelphia, PA 19104. E-mail: lucki@mail.med.upenn.edu