Activity- and pharmacology-dependent modulation of adult neurogenesis in relation to Alzheimer’s disease
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Chapter 5: Comparison of effects of fluoxetine, duloxetine, and running on neurogenesis in female C57Bl6J mice

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Abstract
Hippocampal neurogenesis can be regulated by extrinsic factors, such as exercise and antidepressants. While there is evidence that the serotonin re-uptake inhibitor (SSRI) fluoxetine enhances neurogenesis, the new dual serotonin/norepinephrine reuptake inhibitor (SNRI) duloxetine has not been evaluated in this context. In addition, it is unclear whether effects of antidepressants and running on cell genesis and behavior are of similar magnitude in mice. Here, we assessed neurogenesis and open field behavior in 2 month old female C57Bl/6 mice after 28 days of treatment with either fluoxetine (18 mg/kg), duloxetine (2, 6 or 18 mg/kg) or exercise. New cell survival, as measured by 5-bromo-2’-deoxyuridine (BrdU) labeled cells, was enhanced by 200% in the running group only. Both running and fluoxetine, but not duloxetine, increased the percentage of new cells that became neurons. In the open field test, drug treated animals spent less time in the center than controls and runners. In addition, fluoxetine treatment resulted in reduced locomotor activity. Together, the data shows that the neurogenic response to exercise is much stronger than that produced by antidepressants and implies that the reported anxiolytic effect of these two drugs are unlikely to be mediated by adult neurogenesis in C57Bl/6 mice.
1. **Introduction**

Depression and anxiety disorders are common health problems with 10-20% lifetime prevalence [1]. Antidepressants, such as the SSRI Fluoxetine hydrochloride (fluoxetine) are prescribed to more than 40 million patients worldwide [2]. The mechanism of action for antidepressants as well as the pathophysiology of depression is poorly understood [3]. The observed hippocampal atrophy in depressed patients [4, 5] has raised the possibility that stress-induced reductions in adult hippocampal neurogenesis may in part underlie the condition [6-9]. Additional evidence in support of a link between adult neurogenesis and depression is that antidepressants promote newborn cell proliferation[10, 11] survival and neurogenesis [11-13]. In addition, the 3–4 week therapeutic lag coincides with the maturation time-course of newly born neurons[14] (Jacobs et al., 2000). Furthermore, some classes of antidepressants (tricyclics and SSRIs) reverse depression-like phenotypes in behavioral tests for anxiety, like the novelty suppressed feeding task (NSF) [15].

Similar to pharmacological agents, exercise has anti-depressant and anxiolytic effects [16]. Clinical data from humans shows that running and antidepressants have similar efficacy for treating major depressive disorder [17]. In the hippocampus running increases trophic factor levels [18, 19], angiogenesis [20], dendritic spine density [21] and synaptic plasticity [22]. Specific to the dentate gyrus (DG) subfield of the hippocampus is a robust increase in neurogenesis with exercise [23]. Both running and antidepressants increase BDNF levels [18, 24], which is hypothesized to contribute significantly to neurogenesis and the regulation of mood [25]. In addition, exercise elevates monoamine levels [26] including the precursor for serotonin synthesis, tryptophan hydroxylase [27], which may mediate the reported anti-depressant effect of exercise. Despite the similar behavioral and neurogenic effects of exercise and antidepressants, few direct quantitative and qualitative comparisons have been made previously [24, 28]. Neither has much attention been paid to significant gender differences in prevalence of anxiety; girls are twice as likely as boys to have experienced an anxiety disorder by age 6 [29, 30]. These differences extend through adolescence and into adult populations. Separate epidemiological studies have identified that women are at higher risk for developing anxiety disorders than men [31, 32].
Lifetime prevalence for depression is about 21% in women and 13% in men in the United States[33]. Therefore, we compared effects of two antidepressants and running on neurogenesis and open field behavior, as a test for anxiety in female mice. Specifically, the SSRI fluoxetine and the more potent, new antidepressant duloxetine [34] were evaluated in a chronic four week dosing paradigm and compared to exercise. These two compounds differ in inhibitory constants (Ki) and pharmacokinetics; Duloxetine e.g., is a 4-fold more potent inhibitor of serotonin receptors and preferentially partitions to the brain, while fluoxetine has low blood-brain-barrier penetration [34]. By comparing both fluoxetine and duloxetine, the norepinepherine reuptake inhibition (NRI) pharmacology present in duloxetine could be evaluated. To study potential effects on neurogenesis, the 18 mg/kg duloxetine dose was compared to a previously published 18 mg/kg fluoxetine dose [15] in addition to a 10-fold lower dose (2 mg/kg), and a middle dose (6 mg/kg). Here we show, that in contrast to running, neither the dual-pharmacology anti-depressant Duloxetine nor fluoxetine improved new cell survival whereas fluoxetine did enhance neuronal differentiation of the newly generated cells. These findings suggest a low likelihood that psychotropic effects of SSRI and SNRI compounds are mediated by adult neurogenesis.

2. Results

2.1 Behavior

All of the mice where individually housed at the start of the experiment with one group assigned to running wheel cages (n=6). The average distance run per day was 4.5 ± 0.7 km. Twenty-one days after the start of the experiment mice were tested in open field arenas to evaluate exploratory behavior and anxiety. One way analysis of variance (ANOVA) with repeated measures over time (30 min total, divided into 5 min bins) revealed that there was a significant interaction between distance traveled over time and group (F(5,25) =1.61, p<0.044). Specific post-hoc comparisons showed that the fluoxetine treated mice had significantly reduced overall locomotor activity as compared to controls (p<0.047), and a strong non-significant trend as compared to runners (p=0.057) (Fig. 1a). Furthermore, to
assess anxiety levels the time spent in the center of the open field was measured. One way ANOVA (Group x Center time) revealed a significant difference between the groups ($F_{(5,30)} = 3.08$, $p<0.02$). Post-hoc comparisons showed that fluoxetine treated mice ($p<0.016$) as well as both the duloxetine 6 mg/kg and 18 mg/kg groups ($p<0.05$) spent less time in the center than controls and runners (Fig. 1b). Taken together, the open field data suggest that motor activity is reduced by fluoxetine at a dose of 18 mg/kg and that neither fluoxetine nor duloxetine has anxiolytic effects in female C57Bl/6 mice (Figure 1).
2.2 Survival and phenotype of newborn cells

Mice were injected with bromodeoxyuridine (BrdU) for four days (75 mg/kg) to label dividing cells. BrdU is a thymidine analog incorporated into the DNA of dividing cells during genome replication. This allows BrdU containing nuclei in the DG to be identified through immunohistochemistry. One way ANOVA showed that there was a significant difference between the groups ($F_{(5,30)} = 21.87, p<0.0001$). Specific comparisons showed that runners had a significantly higher number of BrdU positive (BrdU+) cells as compared to all other groups ($p<0.0001$) (Fig. 2e).

In order to establish whether BrdU+ cells had matured to become granule neurons in the DG we co-labeled these cells with mature neuronal nuclei marker (NeuN). Sections were double-labeled with primary antibodies for BrdU and NeuN and incubated with species-specific secondary antibodies conjugated to Alexa-488 and Cy5 fluorophore dyes, respectively. Z-stack images were obtained on a spinning-disk confocal microscope and neurons in the DG were analyzed for the presence of both markers (Fig. 2a-d). Since only the 6 mg/kg dose of duloxetine showed a trend toward increased survival of BrdU+ cells, this was the only duloxetine group to be evaluated for cell fate analysis. One way ANOVA showed a significant difference in the percentage of BrdU/NeuN positive cells between the groups ($F_{(5,30)}=21.87, p<0.0001$). Specific comparisons showed that both fluoxetine ($p<0.003$) and running ($p<0.0006$) significantly increased the population of BrdU/NeuN-positive cells, while there was no effect of duloxetine (Fig. 2f).
Figure 2: The effects of running and antidepressants on cell survival and neurogenesis. Representative photomicrographs (a. control, b. duloxetine 6 mg/kg, c. fluoxetine 18 mg/kg, d. runner) of the SGZ demonstrate the location of BrdU labeled cells (green) within the contour of the DG labeled with NeuN (red). e. Running mice had a more than 2-fold increase in the number of surviving BrdU labeled cells. F. Fluoxetine and running both significantly increased the percentage of BrdU cells colocalizing with the neuronal marker (NeuN). N=6 mice per group, error bars indicate SEM. *p<0.003.
3. Discussion

We studied female C57Bl6 mice to compare the effects of chronic treatments with antidepressants and exercise on parameters of anxiety and neurogenesis. In the open field, the SSRI fluoxetine reduced distance traveled in the open field indicating that locomotor activity was suppressed. Surprisingly, both duloxetine (at doses of 6 and 18 mg/kg) and fluoxetine (18 mg/kg) reduced the amount of time spent in the center of the open field. Regarding new cell survival, only runners had a significant increase in BrdU+ cells as compared to control animals. However, both exercise and fluoxetine enhanced neuronal differentiation. The SNRI duloxetine affected neither cell survival nor neurogenesis. These findings suggested that only some classes of antidepressants can exert a mild neurogenic effect which does not clearly correlate with reduced anxiety in the open field.

Interestingly, there was no effect of fluoxetine on new cell survival. This finding differs from the initial report in male rats [12], but is consistent with research by others in female rats [35]. Our results support previous findings that the neurogenic effects of antidepressants are also indeed hinged upon age. There is evidence that enhanced survival occurs upon administration of fluoxetine in mice that are less than 3 months-old but that aging abolishes this effect on new cell survival [36, 37]. Our study with young mice however, does not show increased BrdU labeling in the C57Bl/6 strain, similar to another study [38]. A side by side comparison of inbred mouse strains found that responses to fluoxetine were dependent on inherent predisposition for serotonin-induced neurogenesis [39]. Separate experiments confirmed that chronic fluoxetine administration did not alter hippocampus neurogenesis in BALB/cj mice [40] or in Sprague-Dawley rats [41]. Altogether the outcome of the present study and work by others indicates that anti-depressants have a mild neurogenic effect that depends on sex, strain, age and pharmacology of the antidepressant.

Upon testing in the open field runners did not differ from controls with regard to distance traveled and the amount of time spent in the center of the field. As running is considered anxiolytic it may be expected that runners would adapt more quickly to the open field and would spend more time in the center. However, in mice there appear to be gender differences in this regard. Male mice show better adaptation and more center entries after two weeks of voluntary wheel running.
Consistent with the results of the present study, in an experiment in which mice ran for 3 months female runners did not differ in open field behavior from controls [43].

Analysis of the effects of antidepressants on open field behavior revealed differences between the SNRI duloxetine and the SSRI fluoxetine. Adaptation to the open field was not changed by treatment with duloxetine. This is consistent with other reports that duloxetine does not affect locomotor activity [44]. Fluoxetine treated mice, on the other hand, showed reduced locomotion in the open field. This finding is in contrast to several reports using a similar dose range that show that fluoxetine increased activity [44, 45]. Other researchers, however, report that a lower dose of fluoxetine (6 mg/kg) reduces the amount of voluntary wheel running [46] and that a 10 mg/kg dose reduces distance traveled in the open field [47]. In addition, treatment with fluoxetine as well as duloxetine at the 6 and 18 mg/kg doses resulted in less time in the center of the field than controls and runners. These findings may be specific to the C57Bl/6 strain as it has been reported that there is only an increase in center time with fluoxetine in the more anxious Balb/C mouse strain [47].

Consistent with previous reports, 4 weeks of voluntary wheel running resulted in a robust enhancement in the survival of newly born cells in the DG of the hippocampus as well as an increase in neurogenesis. This finding has been replicated in different mouse strains, ages and exercise paradigms [23, 48]. The antidepressants, on the other hand, appear to have minimal neurogenic effects. The potent SNRI Duloxetine did not change cell survival or neurogenesis at any of the doses tested. It is possible that this anti-depressant has an entirely different mechanism effect on the factors mediating neurogenesis when compared to fluoxetine. While administration of duloxetine has been associated with a significant increase in BDNF mRNA levels in frontal cortex [49], in the hippocampus only the synaptic compartment shows a change [50]. Fluoxetine, on the other hand results in a significant upregulation of BDNF protein in the hippocampus [51] and was shown to increase neuronal differentiation in the present study, consistent with previous research in mice [37, 38]. However in rats, fluoxetine has not shown induction of BDNF protein[12, 35], indicating that the ability of fluoxetine to induce BDNF may be species-dependent[52].

In summary, the present study shows that exercise has a robust effect on hippocampal neurogenesis, whereas of the two
antidepressants tested only fluoxetine enhanced neuronal differentiation. In addition, antidepressant treated mice spent less time in the center of the open field. These findings suggest there is no close association between the neurogenic and anxiolytic effects of antidepressants in female C57Bl6 mice.

4. Experimental Procedures

4.1 Mice
Female C57Bl6J mice (5 weeks old) were purchased from the Jackson Lab (Bar Harbor, ME). The mice were maintained on a standard NIH-07 diet (Harlan-Tekland, Indianapolis, IN) with free access to water during a 12-hour light/12-hour dark cycle. Two weeks after arrival the animals were housed individually and randomly assigned to control (con), Fluoxetine 18mg/kg (flu 18), and Duloxetine 2/6/18 mg/kg (dul 2/6/18) or running (run) groups. Anti-depressants were obtained from Toronto Research Chemicals (TRC Inc, North York, Ontario, Canada), dissolved in sterile saline, and administered once-daily by subcutaneous injection. Control animals received sterile saline only, injections were performed daily shortly before lights out at 18:00hrs. The running mice were housed with a running wheel and distance run was recorded daily (Clocklab, Coulborn Instruments, Whitehall, PA). In order to analyze newborn cells, BrdU (75 mg/kg) was injected intraperitoneally for the first 4 days. Twenty eight days later, animals were deeply anesthetized by isoflurane inhalation and perfused with phosphate buffered saline. Animals were decapitated and brains were immediately removed. The right hemisphere was washed and placed in 4% paraformaldehyde for immunohistochemistry while the left hemisphere was dissected and frozen on dry ice for biochemical analysis in a separate study. All animal procedures were done in accordance approved by the National Institute of Health Animal Care and Use Committee.

4.2 Open Field Arenas
Animals from all 6 groups were randomized and tested on 2 consecutive days in an open field arena (27.3 x 27.3 cm, height 20.3 cm) ( Med Associates Inc, Georgia, VT). Animals were placed in the center of the arena at the beginning of the testing paradigm and were left undisturbed for 30 minutes. All testing occurred between 08:00 and 11:00 hrs on the day of testing. The center zone was defined as a 10.2
cm square equidistant from the peripheral walls. Each arena had a black floor and walls where x-y movements are monitored by two sets of pulsed-modulated infrared photobeams. This tracking system records data directly to a networked computer and measures ambulatory counts, entries into defined zones, distance traveled, and time spent in each zone (Med Associates Inc., Georgia, VT).

4.3 Bromodeoxyuridine immunohistochemistry

Fixed brain hemispheres were transferred to 30% sucrose in 0.1 M PBS, pH 7.4, until they sank. Brains were then cut coronally in 40 µm serial sections on a fast-freezing sliding microtome and stored in a 96-well plate. A one-in-six series of free floating sections was washed thoroughly in TBS and pre-incubated with 0.6% H₂O₂ for 30 minutes. After rinsing, the sections were incubated in 2N HCl at 37° C for 30 minutes to denature DNA then neutralized in 0.1 M Borate buffer at RT. After thorough washing, the sections were blocked with TBS++ (3% Donkey Serum-0.05 M TBS, 0.5% Triton-X 100) for 30 min at room temperature and incubated with rat anti-BrdU (1: 200, Accurate Chemical Westbury NY) overnight at 4° C. Thereafter, the sections were washed and immersed in biotin-SP-conjugated donkey anti-rat IgG (1: 250, Jackson ImmunoResearch, West Grove, PA) followed by 2 hrs in ABC reagent (1:1000, Vestastain Elite; Vector Laboratories, Burlingame, CA). The sections were then incubated with the substrate 3, 3′-Diaminobenzidine (D4418, Sigma, St. Louis, MO) for 5 min to visualize the cells that had incorporated BrdU. All cells within the granule cell layer and subgranular zone were counted with a 40x objective. Six mid-level dentate gyrus sections were counted from the right hemisphere of each animal and multiplied by 6 corresponding to the 1:6 sampling through the region yielding a standardized total number of BrdU+ cells in the right hemisphere.

4.4 Double immunofluorescence for cell fate analysis

Free floating sections (1:6 series) were simultaneously incubated with primary antibodies against BrdU (1: 100 Accurate Chemical Westbury NY) and NeuN (1:100 Millipore, Billerica, MA). Antibodies were diluted in TBS++ and then sections were incubated for 48 hr at 4° C. After rinses in TBS++ and TBS sections were co-incubated with donkey ant-rat Alexa Fluor 488 (1:250, Molecular Probes, Carlsbad, CA) and donkey anti-mouse Cy3 fluorophore dyes (1:250, Jackson ImmunoResearch, West Grove, PA) for 2 hours at RT. Z-stacked images of cells in the
granule cell layer were imaged on an Olympus IX81 spinning disk confocal microscope (Olympus, Center Valley, PA). At least 50 cells in the DG of each mouse were analyzed for double-labeling between BrdU and the neuronal marker NeuN.

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Activity and pharmacology in young adult mice


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