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Letrozole increases hippocampal neurogenesis in middle-aged female mice

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Abstract

Letrozole, a third-generation aromatase inhibitor, prevents the production of estrogens in the final step in conversion from androgens. Due to its efficacy at suppressing estrogens, letrozole has recently taken favor as a first-line adjuvant treatment for hormone-responsive breast cancer in middle-aged women. Though patient response to letrozole has generally been positive, there is conflicting evidence surrounding its impact on the development of depression. It is possible that letrozole's potential adverse effects on mood are a result of the impact of hormonal fluctuations on neurogenesis in the hippocampus. Thus, to clarify the effects of letrozole on the hippocampus and behavior, we examined how chronic administration affects hippocampal neurogenesis and depressive-like behaviour in middle-aged, intact female mice. Mice were given either letrozole (1mg/kg) or vehicle by injection (ip) daily for 3 weeks. Depressive-like behaviour was assessed during the last 3 days of treatment using the forced swim test, tail suspension test, and sucrose preference test, and the production of new neurons was quantified using the immature neuronal marker, doublecortin (DCX). We found that letrozole increased DCX expression and maturation in the dentate gyrus, but had no significant effect on depressive-like behaviour. Our findings suggest that a reduction in circulating estrogens in middle-aged females increases hippocampal neurogenesis without any adverse impact on behavior; as such, this furthers our understanding of how estrogens modulate neurogenesis, and to the rationale for the utilization of letrozole in the clinical management of breast cancer.

Keywords: Letrozole; estrogens; depression; neurogenesis;

1. Introduction

Estrogen-suppressive therapy is a common and effective adjuvant treatment of hormone-responsive breast cancer in postmenopausal women. Letrozole, a non-steroidal aromatase inhibitor (AI) that prevents the conversion of androgens into estrogens in the final steps of the estrogen-synthesis pathway, is a first-line treatment of choice. Despite its demonstrated benefits, there is conflicting clinical and pre-clinical evidence regarding its adverse effects on mood and cognition. Recently, the effects of letrozole on cognition have attracted more attention, but the evidence for the effects of letrozole on depression is less understood. Both clinical and pre-clinical trials have found opposing effects of letrozole on mood and behaviour (Borbélyová et al., 2017; Chang et al., 2015; Kokras et al., 2018, 2014; Meng et al., 2011). Most animal studies to date have used rodents of varying ages, gonadal hormone status, sex, and duration of treatment, resulting in conflicting data which are poorly understood.

Women are susceptible to developing depression during times of dramatic hormone fluctuations such as the postpartum and perimenopause. Suppression of ovarian hormones can induce a depressive-like phenotype in women and rodents (Frokjaer et al., 2015; Mahmoud et al., 2016), suggesting that a reduction in estrogens renders females more susceptible to depression. Thus, it is possible that the adverse effects of letrozole on mood may be a result of its action to suppress estrogens.

The hippocampus has a high concentration of estrogen receptors, and is a region that is implicated in the pathoetiology of depression. Estrogens modulate adult hippocampal neurogenesis with chronic exposure suppressing neurogenesis independent of its effects to upregulate cell proliferation (Mahmoud et al., 2016). Furthermore, decreased hippocampal neurogenesis is seen in depressed patients and in animal models of depression, which is restored with antidepressant treatment (Mahmoud et al., 2016). Furthermore, androgens enhance hippocampal neurogenesis in adult male rodents (Mahmoud et al. 2016) but it is not known whether androgens can modulate neurogenesis in females. It is possible that changes in neuroplasticity serve as a neural basis for local estrogens to exert their effects on mood. Therefore, we sought to investigate the effects of estrogen suppression due to chronic letrozole treatment on depressive-like behaviour and hippocampal neurogenesis in middle-aged female mice.

2. Methods

2.1 Subjects

Nineteen C57/Bl6J female mice 10-12 months of age were obtained from the Animal Care Centre at the University of British Columbia. All animals were maintained on a 12h light/dark cycle (lights on at 07:00h), group housed (2-3) and given ad libitum access to food (Purina chow) and water. All procedures were performed in accordance with ethical guidelines set by the Canadian Council on Animal Care, and approved by the Animal Care Committee at the University of British Columbia.

2.2 Drug preparation and treatment

All animals received daily intraperitoneal (i.p.) injections of 1mg/kg letrozole or saline vehicle for 21 days (see Fig. 1A; dose chosen due to Mikail et al., 2012). Letrozole was dissolved in 0.9% saline at 0.1mg/mL, dissolved with aid of ultrasonic bath.

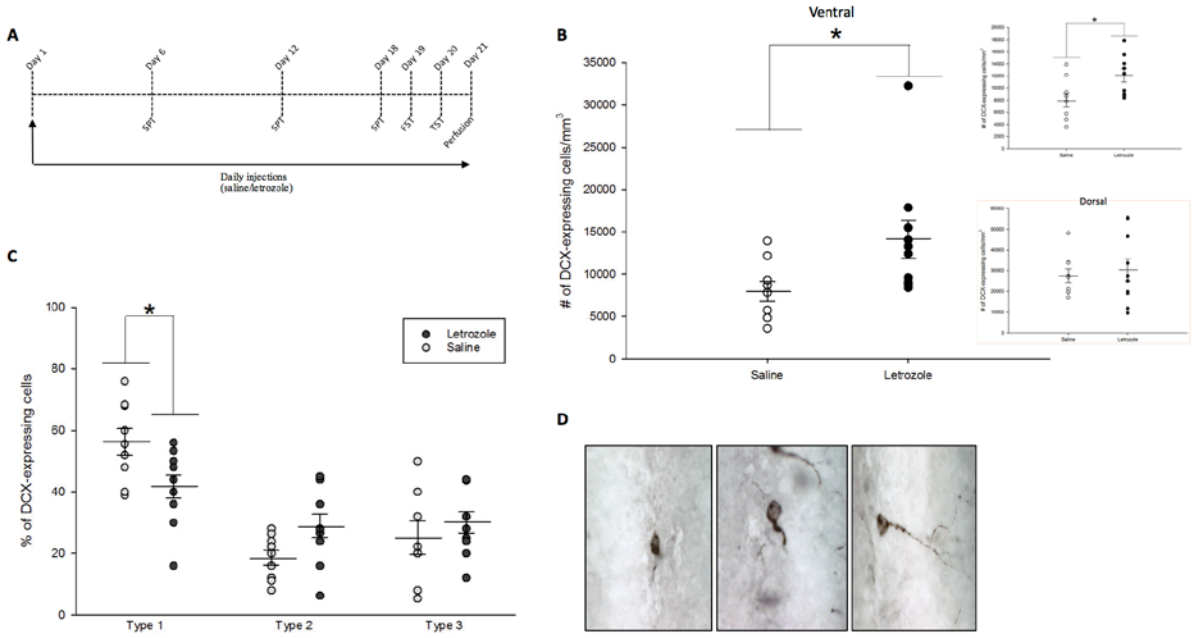

Figure 1: A) Experimental timeline. B) Mean density of doublecortin (DCX)-expressing) cells in the ventral dentate gyrus (DG). Letrozole significantly increased the density of DCX-expressing cells in the ventral dentate gyrus(DG). Inset (top): DCX-expressing cells in the ventral DG without outlier >2SD away from the mean. Inset (bottom): Mean number of DCX-expressing cells in the dorsal DG. C) Percentage of DCX-expressing cells in the ventral DG in each maturational stage. Letrozole decreased the proportion of type 1(proliferative) DCX-expressing cells compared to controls, and tended to increase the proportion of type 2 (intermediate) DCX-expressing cells ($p<0.06$). D) Representative photomicrographs of DCX-expressing cells, viewed at 400X magnification. From left to right: type 1 cell, proliferative; type 2 cell, intermediate; type 3 cell, post-mitotic. *$p<0.05$. ± SEM.

2.3 Behavioural Testing

Behavioural Testing occurred during days 18-20 of 21 days of letrozole or saline treatment, with the exception of the Sucrose Preference Test which was administered weekly.

Forced Swim Test (FST) and Tail Suspension Test (TST)

FST and TST were conducted as described previously (Saeedi Saravi et al., 2016). Each mouse was subjected to a single 6-minute FST or TST session. FST was conducted in a vertical glass beaker (30cm height x 20cm diameter) filled with clean water (24°C) at a depth of 15cm. In the TST session, mice were suspended by their tails above the ground with a 17cm strip of tape within a 3-walled rectangular chamber. Both tests were videotaped and scored using BEST collection software (Educational Consulting, Hobe Sound, FL, USA) by an individual blind to treatment condition. Percent time spent in mobile and immobile behaviours were analyzed.

Sucrose Preference Test (SPT)

Each mouse was habituated to a 1% sucrose solution and the two-bottle procedure by introducing two identical bottles with water and 1% sucrose (counterbalanced) into their home cage for a 48h period. After acclimatization, the test was administered for three days before the start of treatment (baseline) and then once a week for 3 weeks over the course of letrozole treatment. Briefly, mice were single housed and

simultaneously food and water deprived for 4h. Mice were then presented with 2 bottles for 12h between 20:00h and 08:00h, after which they were re-paired with cage mates. Sucrose preference was calculated using the formula: sucrose preference=(sucrose consumed/(sucrose + water consumed))x100.

2.4 Tissue Collection

24h after FST, mice were given an overdose of sodium pentobarbital, and blood was collected by cardiac puncture. Mice were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were extracted and post-fixed in paraformaldehyde overnight at 4°C. Brains were transferred to 30% sucrose and stored at 4°C. Brains were sliced in 30µm coronal sections using a Leica SM2000R microtome (Richmond Hill, Ontario, Canada). Sections were stored in antifreeze (20% glycerol and 30% ethylene glycol in 0.1M PBS) at -20°C until processing.

2.5 Doublecortin (DCX) Immunohistochemistry

Sections were rinsed in phosphate buffered saline (PBS) and treated with 0.6% hydrogen peroxide in dH20 for 30 minutes. Sections were rinsed and incubated for 24h at 4°C in primary antibody solution: 1:1000 goat anti-doublecortin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 0.04% Triton-X in PBS, and 3% normal rabbit serum. Sections were then rinsed and incubated in secondary antibody solution for 24h at 4°C: 1:1000 rabbit anti-goat (Vector Laboratories, Burlington, ON, Canada) in 0.1M PBS. Then, sections were rinsed and incubated in an avidin-biotin complex (ABC Elite Kit, 1:1000, Vector Laboratories) in PBS for 2hr. Sections were rinsed and subsequently 2 x 2min in 0.175M sodium acetate buffer. Immunoreactants were visualized using diaminobenzadine (DAB) in the presence of nickel (DAB peroxidase substrate kit, Vector), mounted on slides, dried, dehydrated and coverslipped.

2.6 Microscopy, cell quantification, and cell phenotyping

An investigator blinded to treatment condition quantified DCX–expressing cells and analyzed cell morphology. DCX- expressing cells were quantified in the granule cell layer of the dentate gyrus in every 10^{th} section along the rostral-caudal axis, using the 40x objective on an Olympus CX22LED brightfield microscope. Raw counts were multiplied by 10 to get an estimate of the total number of DCX-expressing cells, separately in dorsal and ventral regions. Areas of the granule cell layer were quantified using ImageJ (NIH, Bethseda, MD) and used for density calculations (number of cells per mm^2).

DCX phenotypes (Figure 1 C) were analyzed using the 100× objective on an Olympus CX22LED brightfield microscope. 50 DCX-expressing cells (25 dorsal GCL and 25 ventral GCL) were randomly selected for each animal, and categorized into one of three maturational stages based on previously established criteria (Snyder et al., 2009): proliferative (no process or short process), intermediate (medium process with no branching), or post-mitotic (long processes with branching into the GCL and molecular layer).

2.7 Data Analyses

All statistical analyses were performed using Statistica software (Tulsa, OK). Behavioural tests (TST, FST), density of DCX-expressing cells, and morphology of DCX-expressing cells were each analyzed using repeated measures analysis of variance (ANOVA) with drug treatment (Letrozole or Vehicle) as the between-subjects factor and behavior (immobile, mobile), region (dorsal, ventral) or cell type (type 1,2,3) as within-subjects factor with age as a covariate. Uterine and adrenal mass were analyzed using a student's t-test. Percent sucrose preference and percent change in body mass were analyzed with a repeated measures ANOVA with drug treatment as the between-subjects factor and week

as the within-subjects factor. Post-hoc analyses used the Newman-Keuls test, a priori tests utilized Bonferroni corrections.

3. Results

3.1 Letrozole upregulated the density of immature neurons in the dentate gyrus

Letrozole treatment significantly increased the density of DCX-expressing cells (main effect of treatment ($F(1,16)=8.12$, $p<0.011$, $\eta_p^2= 0.34$). Indeed, letrozole treatment increased the density of DCX-expressing cells in the ventral ($p=0.004$; cohen's $d=1.09$; figure 1B) more so than the dorsal region ($p=0.06$, Cohen's $d=0.20$). Overall there were more DCX-expressing cells in the dorsal compared to the ventral region (main effect of region: $p=0.02$, $\eta_p^2= 0.22$).

3.2 Letrozole decreased the proportion of proliferative DCX-expressing cells and increased the proportion of more mature DCX-expressing cells in the ventral dentate gyrus

A priori analysis revealed letrozole decreased the proportion of proliferative (type 1) DCX-expressing cells compared to saline in the ventral region ($p<0.012$; Cohen's $d=1.17$; figure 1e) but not the dorsal region ($p= 0.77$; Cohen's $d=0.12$), There was a trend for letrozole to increase the proportion of type 2 DCX-expressing cells ($p=0.06$) but no other significant effects (P's >0.2).

3.3 Letrozole had no significant effect on behavior in the SPT, TST, and FST

In the FST, TST, or SPT there were no significant differences in behaviors between groups (all p's < 0.15, figure 2A-C).

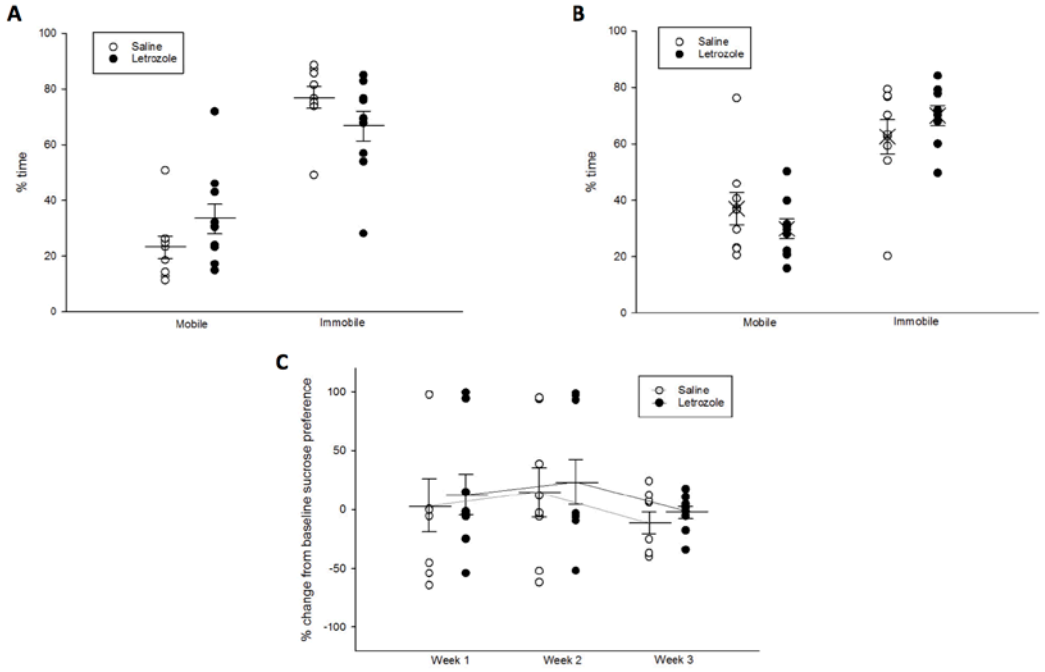

Figure 2 A-C: Letrozole had no significant effect on any measure (all ps>0.1). A) Percent time spent mobile and immobile in the forced swim test. B) Percent time spent mobile and immobile in the tail suspension test. C) Percent change from baseline sucrose preference over 3 weeks of the sucrose preference test. ±SEM.

3.4 Letrozole significantly decreased uterine mass

Letrozole-treated mice had significantly lower uterine mass (0.045g ±0.005) than controls (0.06g ±0.005; $t(17)=2.55$, $p=0.02$; Cohen's $d=0.966$), but no significant effect of letrozole on body or adrenal mass (p's < 0.11).

4. Discussion

We found that letrozole increased the density of immature neurons in the ventral dentate gyrus of middle aged females. This is consistent with findings that long-term ovariectomy in middle-aged female rats increased, while estrogens decreased the survival of immature neurons (Barha et al., 2015). Additionally, we found that letrozole lowered the proportion of the least mature DCX-expressing neurons. This suggests that letrozole increased the rate of maturation of immature neurons, promoting survival past the proliferative phase into the intermediate and post-mitotic phase, consistent with a trend for letrozole to increase type 2 DCX-expressing cells. As DCX is expressed for 28 days in mice (Snyder et al., 2009), this would have entailed that some of the more mature cells were produced without letrozole exposure. Future studies should determine the impact of chronic letrozole on cell proliferation as well as survival of new neurons independent of effects on cell proliferation.

Interestingly, the effects of letrozole to influence neurogenesis were exclusively in the ventral hippocampus. The ventral hippocampus is implicated in modulating stress and affect (Fanselow and Dong, 2010). Given this, coupled with our lack of findings on affective behavioural measures, it suggests neural consequences of letrozole may be seen prior to any behavioural changes.

Chronic letrozole had no significant effect on measures of depressive-like behavior (FST,TST, SPT), in the present study, consistent with other studies examining immobility in the FST (Kokras et al., 2018, 2014; Meng et al., 2011). However, acute letrozole in young, ovariectomized female rats had an antidepressant effect in the FST, while chronic letrozole had no such effect (Kokras et al., 2014). Chronic letrozole in young, ovariectomized mice increased anxiety in the open field test and elevated plus maze (Meng et al., 2011) while chronic letrozole in middle-aged cycling female rats had no effect on anxiety in the elevated plus maze (Borbélyová et al., 2017). Collectively these results suggest that chronic letrozole treatment has no significant effect on depressive-like behaviour in middle-aged females. Future studies should consider the effects of letrozole on depressive-like behavior in the context of a challenge (chronic stress, cancer) in which more robust effects on behavior may be seen.

It is possible that an increase in testosterone that accompanies aromatase inhibition is responsible for mitigating negative behavioural effects of estrogen depletion. Castratrated male rats are more susceptible to developing depressive-like endophenotypes following chronic stress (Wainwright et al., 2011). Similarly, in men, hypogonadism is associated with depressive symptoms, and androgen treatment can ameliorate these symptoms (Zarrouf et al., 2009). Furthermore, testosterone given to surgically menopausal women can elevate mood (Sherwin, 1988). Further studies could evaluate the role of testosterone on mood after aromatase inhibition in middle-aged females.

5. Conclusion

We demonstrate that in middle-aged female mice, chronic letrozole increased hippocampal neurogenesis but had no effect on depressive-like behavior. Further studies investigating the mechanisms behind potential neuropsychiatric effects of aromatase inhibitors on middle-aged women may shed light on the impact of adjuvant cancer treatments on quality of life.

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