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Chronic Fluoxetine Dissociates Contextual from Auditory Fear Memory

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Abstract

Fluoxetine is a medication used to treat Major Depressive Disorder and other psychiatric conditions. These experiments studied the effects of chronic fluoxetine treatment on the contextual versus auditory fear memory of mice. We found that chronic fluoxetine treatment of adult mice impaired their contextual fear memory, but spared auditory fear memory. Hippocampal perineuronal nets, which are involved in contextual fear memory plasticity, were unaltered by fluoxetine treatment. These data point to a selective inability to form contextual fear memory as a result of fluoxetine treatment, and they suggest that a blunting of hippocampal-mediated aversive memory may be a therapeutic action for this medication.

Keywords

fluoxetine; depression; fear; context; memory

Introduction

Over 350 million people struggle with Major Depressive Disorder (MDD), a condition characterized by excessive guilt and decreased energy, mood and appetite. Severe MDD may also result in suicidal thoughts and psychotic symptoms [1]. Added to these symptoms, MDD is characterized by a bias towards negative memories [2–5] and to negative autobiographical narratives that are sub-served by the hippocampus [6–8]. Selective serotonin reuptake inhibitor (SSRI) treatment has therapeutic effects on this memory bias [9].

Based upon these clinical findings we sought to study the effects of the SSRI, fluoxetine, on negative memories mediated by the hippocampus. To study this we examined the effects of chronic fluoxetine on contextual fear memory, a form of aversive learning where the

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Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

hippocampus is considered to play a central role [10]. During contextual fear memory rodents form memories of foot shock (unconditioned stimulus; US) associated with multisensory cues (conditioned stimulus; CS).

To determine whether chronic fluoxetine has effects that are specific to contextual fear memory we also studied its effects on auditory fear memory. In auditory fear memory an association is formed between the US and an auditory cue (CS). This form of learning is thought to be primarily mediated by the amygdala [11].

Our studies further examined the effects of chronic fluoxetine on perineuronal nets (PNNs). PNNs are structures consisting of chondroitin sulfate proteoglycans that organize around neurons during brain development [12]. The disruption of PNNs in the mature hippocampus alters contextual fear memory [13]. In addition, fluoxetine alters PNN density in brain circuitry [14]. Therefore, these studies also included an examination of PNNs in the CA1 and DG areas of the hippocampal region.

Our studies found that that chronic fluoxetine treatment of adult mice impaired their contextual fear memory, but spared auditory fear memory. These data point to a selective inability to form contextual fear memory as a result of fluoxetine treatment, and they suggest that a blunting of hippocampal-mediated aversive memory processes may be a therapeutic action for this medication. At a histological level, hippocampal PNNs were unaltered by fluoxetine treatment. Although not reflected in PNN density our behavioral findings parallel other studies demonstrating fluoxetine's ability to alter hippocampal-related plasticity [15, 16].

Methods

Animals

C57BL/6J mice were bred in our colony maintained at The Scripps Research Institute. These experiments were conducted with male mice and drug treatments were started at ~2 months of age. This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by The Scripps Research Institute guidelines for the humane care and use of laboratory animals and all efforts were made to minimize suffering.

Drug Treatment

Mice were treated with fluoxetine (Eli Lilly), at 0.16mg/ml in their drinking water. Our measurements of daily water intake estimated this dose to result in fluoxetine treatment of 19 mg/kg per day. Based upon prior studies of plasma fluoxetine this administration would approximate levels in the upper end of fluoxetine's therapeutic range of 20–80mg daily [17, 18]. We chose this dose since it represents fluoxetine levels that are used to target severe and oftentimes treatment resistant depressive symptoms [19, 20]. The dose is also similar to that used in other studies that have examined fluoxetine's effects on brain plasticity [15, 21]

Chronic fluoxetine treatment has been defined as between 24–30 days according to prior studies [16, 21, 22]. In Experiment 1 mice received fluoxetine for 30 days. In Experiment 2

mice received fluoxetine for 24 days. Since fluoxetine is photosensitive, bottles were wrapped in aluminum foil and medication was replaced every 7–10 days. Intake was monitored weekly to ensure regular consumption and comparable intake between experimental groups. Control animals received drinking water alone.

Behavior

The first experiment used a between-subject design to study the contextual fear memory of one fluoxetine treated group, compared to the auditory fear memory of a second fluoxetine treated group. Fluoxetine-treated mice received the medication in their drinking water for 30 days prior to fear conditioning and continuously up until memory retrieval.

To examine contextual fear memory, fluoxetine-treated mice (n=6) and drinking water controls (n=8), underwent contextual fear conditioning in Context A. Context A consisted of a wintergreen scented square chamber with black and white checkerboard pattern and aluminum walls (30-cm length × 24 cm width) and a grid floor that delivered footshock (FreezeFrame). Contextual fear conditioning consisted of 109 seconds of free exploration followed by four non-signaled footshocks (duration 1s, intensity 1 mA) with an inter-stimulus interval of 70s. The total duration of training in Context A was 380s. Twenty four later contextually fear conditioned animals were re-exposed to Context A for 120 seconds and freezing levels measured.

To examine auditory fear memory, a separate group of fluoxetine-treated mice (n=7) or drinking water controls (n=6) underwent auditory fear conditioning in Context A. Animals were fear conditioned in Context A, as described above, but with white noise (10s, 85 dB 2800 Hz) preceding and co-terminating with each shock. Twenty four hours later, auditory fear memory was retrieved within Context B. Context B consisted of an opaque plastic container whose floor was covered with sani-chips (Allentown caging, base: 20-cm length×12-cm width, top: 22-cm length×14-cm width). This container sat within a larger, lemon-scented fear conditioning chamber (30-cm length × 24-cm width). Mice were placed in the opaque plastic container and their freezing was measured during 12, 10 second exposures to white noise.

The second experiment used a within-subjects design to study the contextual and auditory fear memory of a single fluoxetine treated group. Fluoxetine-treated mice received the medication in their drinking water for 24 days prior to fear conditioning and continuously up until retrieval. Fluoxetine-treated mice (n=8) or drinking water controls (n=8) were subject to auditory fear conditioning in Context A. Twenty four hours later, either contextual or auditory fear memory were tested in Context A or B, respectively, using a counterbalanced design. 48 hours after the original fear conditioning, animals tested for contextual fear memory were then tested for auditory fear memory and vice versa.

Perineuronal nets (PNNs)

PNNs were measured in mice exposed to fluoxetine for 30 days (n=4) or in mice who received drinking water (n=4). PNNs were visualized according to published methods [23, 24]. Briefly, animals were perfused with 4% paraformaldehyde (PFA) in PBS. Brains were postfixed in 4% PFA and then placed in 30% sucrose. Brain sections were collected with a

vibratome in ice cold PBS. Sections were incubated in a blocking solution of 3% BSA and 0.2% Triton-X-100 in PBS, pH 7.4, and then incubated in a solution of biotin-conjugated lectin wisteria floribunda agglutinin (WFA) (10 µg/ml). WFA was detected using FITC conjugated streptavidin (10 µg/ml in PBS). Images were collected with a fluorescence microscope and PNNs were counted in the CA1 region and dentate gyrus (DG) by two raters blind to experimental treatment.

Statistics

Freezing during white noise exposures was totaled for each animal. The effects of fluoxetine on contextual versus auditory freezing were then analyzed with a 2-Way ANOVA or repeated measures ANOVA. A Tukey method for multiple comparisons post-hoc test was used to examine data points with significant differences. PNN data was analyzed with a Student's t-test.

Results

In the first experiment fluoxetine-treated mice underwent either auditory or contextual fear conditioning. Twenty four hours later, fear memory was retrieved with either auditory cues or contextual cues, respectively. Fluoxetine-treated mice showed no difference in their freezing to auditory cues compared to controls. In contrast, fluoxetine-treated animals showed dramatic reductions in their contextual fear memory compared to controls (Figure 1). An ANOVA found a main effect for retrieval cue ($F(1,23)=70.33$, $p<0.0001$) and fluoxetine ($F(1,23)=22.63$, $p<0.0001$), and a significant interaction (retrieval cue $\times$ fluoxetine $F(1,23)=15.93$, $p<0.001$). Post-hoc testing found a significant decrease in contextual freezing for the fluoxetine-treated group compared to the contextual freezing of the control group ($p<0.001$). (Figure 1).

In the second experiment the contextual and auditory fear memory of a single fluoxetine-treated group of mice was tested. Fluoxetine-treated mice underwent auditory fear conditioning. Since this involves exposure to a novel context, the training box, both auditory and contextual associations are formed. Twenty-four and forty eight hours later, contextual and auditory fear memory were tested in a counterbalanced design. The freezing of control mice was slightly less in our second experiment, but still within a variance range commonly seen in studies of fear memory [13, 25]. Fluoxetine-treated animals again showed no difference in their freezing to auditory cues compared to controls (Figure 2). However, consistent with the first experiment, fluoxetine-treated animals showed significant reductions in their contextual fear memory compared to controls. A repeated measures ANOVA found a significant interaction (retrieval cue $\times$ fluoxetine: $F(1,12)=8.282$, $p<0.05$) and an insignificant effect for counterbalancing order ($p=0.600$) (Figure 2). Post-hoc testing found a significant decrease in contextual freezing for the fluoxetine-treated group compared to the control group ($p<0.05$). In contrast, auditory freezing was not affected in the fluoxetine-treated mice (Figure 2). The enzymatic degradation of PNNs in the hippocampus selectively impairs contextual fear memory [13], resembling the very phenotype that was uncovered with fluoxetine-treatment. Added to these observations are data showing that fluoxetine alters PNN density [14]. Based upon these observations it was hypothesized that fluoxetine

might decrease PNNs in the CA1 and DG region as a potential mechanism for its effects on contextual fear memory.

Within the hippocampus, ~15 PNNs per CA1 region and ~5 PNNs per DG region were measured in control animals. These values fall within the range of CA1 and DG PNN density assayed in other studies. [26, 27]. Despite the dramatic behavioral effects of chronic fluoxetine there was not a change in PNN density within the hippocampal CA1 or DG region of fluoxetine-treated animals (Figure 3).

Discussion

Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) and 5-HT_{2C} receptor antagonist that is commonly prescribed in the treatment of MDD and other psychiatric conditions [28]. In addition to treating MDD in adults and children [29], fluoxetine is the only FDA approved medication for the treatment of bulimia nervosa and obsessive compulsive disorder [30, 31].

These experiments tested the effects of fluoxetine on fear memory. While the serotonergic modulation of fear memory has been extensively studied [27, 32–37], these experiments are the first to examine the effects of chronic, preconditioning fluoxetine on both the contextual and auditory fear memory of mice. Using two differing experimental paradigms, the fluoxetine treatment of adult mice impaired their contextual fear memory, but spared auditory fear memory.

These results are consistent with decreased contextual freezing in rats treated with SSRIs [33–36]. In contrast, auditory freezing tends to be increased or unaltered by SSRI treatment [27, 34, 37, 38]. In these previous studies, decreased contextual freezing was attributed to an anxiolytic effect for the medication [33–36]. An anxiolytic effect would, however, be expected to decrease freezing to both tone and context. The current study's direct comparison of each pinpoints a selective freezing deficit to context. This result suggests a selective inability to form contextual fear memory.

A similar dissociation between contextual and auditory fear memory occurs in two other experimental scenarios. In adult rodents, lesioning the hippocampus results in a similar selective contextual fear memory deficit [10]. Based upon these findings the hippocampus is considered to play a central role in mediating contextual fear memory [10]. There is also a selective impairment in contextual fear memory in developing rats [39, 40] and mice [41]. The close parallel between this developmental behavior, and the effects of hippocampal damage, suggest that the immature phenotype may be due to a delayed maturation of this brain structure [40, 41].

Given the role of the hippocampus in contextual fear memory, our experiments also assessed whether fluoxetine treatment affected PNNs in this brain structure. This analysis was performed since the degradation of PNNs in the adult hippocampus impairs contextual fear memory but leaves auditory fear memory intact [18]. These effects are neuroanatomically specific to the hippocampus since PNN degradation in neither the amygdala or medial prefrontal cortex affects contextual fear memories [13, 24]. Added to these findings are data showing that fluoxetine decreases the density of PNNs in the CA1 and DG region of the

hippocampal circuit when delivered at certain developmental stages [14]. These combined observations led to the hypothesis that PNN reorganization could underlie fluoxetine's contextual fear memory effects in this study.

Contrary to this hypothesis, CA1 and DG PNN density were not affected by four weeks of fluoxetine treatment. This finding differs from studies reporting that developmental fluoxetine treatment decreases CA1 and DG PNN density [14]. However, the results are similar to those that find that adult fluoxetine treatment leaves CA1 PNN density unaffected [27]. Taken together these data indicate that fluoxetine may have differential effects on PNN density when it is delivered to developing versus adult animals.

Although not reflected in PNN density, our behavioral findings parallel other histological studies demonstrating fluoxetine's ability to alter the plasticity of the hippocampus. While not measured in this study, fluoxetine reduces the percentage of parvalbumin-positive neurons surrounded by PNNs in the CA1 region, suggesting that they become more immature [27]. Within the dentate gyrus fluoxetine causes a downregulation of mature granule cell markers in association with immature functional characteristics [15]. Hippocampal neurogenesis is also result of fluoxetine treatment [16]. Hippocampal neurogenesis could be a mechanism for memory impairment caused by fluoxetine since neurogenesis contributes to memory impairment in mice [42].

Fluoxetine's effects may also be due to direct effects on the serotonergic regulation of fear circuitry. Stimulating auto-inhibitory 5-HT_{1A} receptors in the median raphe nucleus also causes decreased contextual freezing [43, 44]. Therefore, as previously proposed [32], it may be that SSRI treatment increases synaptic serotonin levels to stimulate 5-HT_{1A}, and to decrease contextual freezing. Alternatively, fluoxetine is a 5-HT_{2C} receptor antagonist [28]. 5-HT_{2C} antagonism might contribute to memory impairment in our experiments since 5-HT_{2C} mutations impair dentate gyrus function [45], and 5-HT_{2C} blockade impairs the consolidation of fear memory [46].

This study has limitations. It is possible that fluoxetine increases baseline locomotor activity which could artificially decrease contextual freezing measures. We believe this is unlikely, though, since fluoxetine has been given to C57BL/6J mice at a similar dose and duration and was found to decrease locomotion [47]. Indeed, a decrease, or at least no effect on locomotion, has been reported in additional prior studies of chronic fluoxetine treatment in mice [22, 48]. Furthermore, if increased locomotor activity or other confounding drug effects were important variables, they might be expected to have generalized effects on both auditory and contextual fear memory. Using both a between and within-subjects design we show that the fluoxetine treated animals have a specific deficit in contextual but not auditory freezing. This specificity suggests that the memory impairment is not an artifact.

Another limitation is that these experiments did not test the effects of fluoxetine in an animal model of depression. Abnormally high contextual fear learning and memory are observed in some rodent models of depression [49, 50]. Studying fluoxetine's ability to modulate high contextual fear in these models may be a way to broaden an analysis of its mechanisms.

In summary, these experiments find a selective inability to form contextual fear memory as a result of fluoxetine treatment. They suggest that a blunting of hippocampal-mediated aversive memory processes may be a therapeutic action for this medication. A predisposition towards negative memories sub-served by the hippocampus is found in patients with MDD [2–7]. Antidepressants have therapeutic effects on this memory bias [9], and also have selective effects on contextual anxiety but not cued fear in humans [51]. This paper replicates this shift in an animal model that may help to elucidate novel and underappreciated actions for SSRI treatment.

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Abbreviations

MDD	Major Depressive Disorder
SSRI	selective-serotonin reuptake inhibitor
CS	conditioned stimulus
US	unconditioned stimulus
PNN	perineuronal net
DG	dentate gyrus
WFA	wisteria floribunda agglutinin
PBS	phosphate buffered saline
PFA	paraformaldehyde

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Highlights

- ▶ Fluoxetine treatment of adult mice impaired their contextual fear memory but spared auditory fear memory.
- ▶ These data suggest that a blunting of hippocampal-mediated aversive memory may be a therapeutic action for this medication.
- ▶ Hippocampal perineuronal net (PNN) density was not affected by fluoxetine

Fig. 1. Fluoxetine selectively impaired contextual fear memory using a between-subjects experimental design

Mice treated with fluoxetine for ~4 weeks prior to fear conditioning show a selective impairment in contextual fear memory 24 hrs after fear conditioning. (** $p < 0.001$).

Fig. 2. Fluoxetine selectively impaired contextual fear memory using a within-subjects experimental design

Mice treated with fluoxetine for ~4 weeks prior to fear conditioning show a selective impairment in contextual fear memory 24–48 hrs after fear conditioning. (* $p < 0.05$).

Fig. 3. PNNs in the hippocampus were unaltered by chronic fluoxetine treatment

A) Representative fluorescence microscopy image of PNNs in the CA1 region of the hippocampus. Scale bar = 100 μ m. **B)** CA1 and DG PNN density were not affected by fluoxetine. PNN= perineuronal net, DG=dentate gyrus.