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Adverse effects of aromatase inhibition on the brain and behavior in a non-human primate

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Adverse effects of aromatase inhibition on the brain and behavior in a non-human primate

Abbreviated title: Adverse effects of Letrozole on brain and behavior

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Abstract: Breast cancer patients using aromatase inhibitors (AIs) as an adjuvant therapy often report side effects including hot flashes, mood changes, and cognitive impairment. Despite long-term use in humans, little is known about the effects of continuous AI administration on the brain and cognition. We used a primate model of human cognitive aging, the common marmoset, to examine the effects of a 4-week daily administration of the AI Letrozole (20 µg, p.o.) on cognition, anxiety, thermoregulation, brain estrogen content, and hippocampal pyramidal cell physiology. Letrozole treatment was administered to both male and female marmosets and reduced peripheral levels of estradiol (E2), but unexpectedly increased E2 levels in the hippocampus. Spatial working memory and intrinsic excitability of hippocampal neurons were negatively affected by the treatment possibly due to increased hippocampal E2. While no changes in hypothalamic E2 were observed, thermoregulation was disrupted by Letrozole in females only, indicating some impact on hypothalamic activity. These findings suggest adverse effects of AIs on the primate brain and call for new therapies that effectively prevent breast cancer recurrence while minimizing side effects that further compromise quality of life.

Significance

Aromatase inhibitors (AIs) are used as an adjuvant therapy for estrogen-receptor positive (ER+) breast cancer and are associated with side effects including hot flashes, depression/anxiety, and memory deficits severe enough for many women to discontinue this life-saving treatment. AIs are also used by men, yet sex differences in the reported side effects have not been systematically studied. We show that AI-treated male and female marmosets exhibit behavioral changes consistent with these CNS symptoms, and elevated hippocampal estradiol and compromised hippocampal physiology. These findings illustrate the need for 1) a greater understanding of the precise mechanisms by which AIs impact brain function, and 2) the development of new treatment approaches for breast cancer patients that minimize adverse effects on the brain.

Introduction

Estrogens are synthesized by the conversion of testosterone (T) through the enzyme aromatase. To prevent this conversion, women with estrogen receptor-dependent breast cancers are often given aromatase inhibitors (AIs) as an adjuvant therapy for many years (Burstein et al., 2014; Chumsri et al., 2016). AIs are associated with side-effects that impair quality of life, including insomnia (Desai et al., 2013), hot flashes (Rand et al., 2011), depression (Chumsri et al., 2016) and memory deficits (Blaustein, 2017; Bender et al., 2007*;* but see Ganz et al., 2013). For example, reduced hippocampal (HPC) activity and increased prefrontal (PFC) activity along with slightly diminished memory was reported in women taking Letrozole (Bayer et al., 2015). Memory deficits in women have also been reported in other studies (e.g. Collins et al., 2009), but not all (for a review, see Lee et al., 2016), and one study in older men showed improved spatial memory following AI treatment (Cherrier et al., 2005). However, the mechanisms by which AIs give rise to these CNS symptoms remain unclear as studies in humans often lack proper controls and are hampered by confounds such as concurrent chemotherapy/radiotherapy treatment, stress, and disease stage. AI use is also relevant in men with prostate (Dias et al., 2016) and breast cancer (Fentiman, 2018), yet no systematic analysis of sex differences has been conducted on the above-mentioned side effects.

Experimental studies in appropriate animal models are needed to complement those in humans as they offer the advantage of including appropriate control groups in the absence of the confounds listed above. Aromatase is expressed in several brain regions (Cornil, 2017; Naftolin, Ryan, & Pierto, 1972), including those involved in processes regulating thermoregulation (i.e. hypothalamus; Roselli, 2013), emotion (i.e. amygdala; Roselli, 2013), and cognition (HPC, cortex; Hojo et al., 2004; Vahaba & Remage-Healey, 2015). Evidence from rodent and bird studies suggest that aromatase inhibition results in HPC-dependent memory deficits. For example, aromatase knock-out (ArKO) male and female mice show impaired spatial

reference memory compared to wild type (Martin et al., 2003); inhibiting aromatase in the HPC reduces novelty preference in ovariectomized (OVX) female mice (Tuscher et al., 2016) and decreases spatial learning and memory in male zebra finches (e.g. Bailey et al., 2017). These effects may be due to synaptic changes in the HPC, as E2-synthesis inhibition reduces the number of spine synapses in the HPC in vitro (Kretz, 2004) and in vivo in OVX mice (Zhou et al., 2010), potentially by destabilizing the spine cytoskeleton (Vierk, Brandt, & Rune, 2014). Aromatase inhibition in HPC slices also results in a failure to induce LTP in female, but not male mice (Vierk et al., 2012). These studies have focused on HPC-dependent memory and physiology, without addressing potential changes in thermoregulation and mood. Further, these studies were conducted in animal models phylogenetically distant from humans.

The aim of the present study was to determine whether continuous neuroestradiol synthesis inhibition in a nonhuman primate, the common marmoset, produces adverse effects similar to those reported in humans. The structural and functional organization of the marmoset brain is comparable to the rhesus or human brain (Chaplin et al., 2013). Marmosets also share many similarities with humans in sleep and thermoregulation patterns (Hoffmann et al., 2012; Gervais et al., 2016), cognitive ability (Spinelli et al., 2004; Yamazaki et al., 2016), and anxiety profiles (Barros et al., 2008; Galvao-Coelho et al., 2008), implicating this species as a strong translational model for studying AI effects on the CNS. Aromatase is expressed in the HPC of marmosets (Wehrenberg, Prange-Kiel, & Rune, 2001), suggesting that local estrogen synthesis occurs in this primate, as in humans (as reviewed by Azcoitia, Yague, Garcia-Segura, 2011). We hypothesized adverse effects of daily AI treatment on the brain, behavior and memory, including reduced excitability of CA1 neurons, increased anxiety, thermodysregulation, and memory impairment. When possible, attempts were made to identify sex differences.

Materials and Methods

Subjects

Sixteen (males: *n* = 9; females: *n* = 7) middle- to older-aged common marmosets (*Callithrix jacchus;* age: 5.5-9.5 years old) weighing 332-511 g participated in the study. Marmosets have an average lifespan of about 12 years and show signs of aging by the age of 8 (Tardif et al., 2011). They were gonadectomized (GDX) approximately three years prior to the start of the experiment. The marmosets were housed in opposite-sex pairs (*n* = 14), except two males that were housed together. The cages were made of stainless-steel mesh (101 X 76.2 X 78.74 cm) and contained perches, platforms, one nest box, and hammocks to promote species-typical behavior including foraging, scent-marking, and climbing. Animals were maintained under a 12-h light cycle (lights on at 8:30 AM), and the ambient temperature set at approximately 27ºC, and humidity at around 50%. Marmosets were fed Mazuri Callitrichid High Fiber Diet 5M16 (Purina Mills, St Louis, MO) supplemented with a variety of fresh fruit, nuts, and mealworms. Fruit and nuts were provided twice daily (8-9 AM and 1-3 PM) and water was available *ad libitum*. The monkeys were provided with daily enrichment, including foraging tubes and a variety of toys. The animals were cared for in accordance with the guidelines published in the Guide for the Care and Use of Laboratory Animals, 8th edition. The studies were approved by the University of Massachusetts Institutional Animal Care and Use Committee.

Experimental design

Behavioral, spatial memory performance and facial temperature measures were collected to address whether continuous administration of oral Letrozole to middle-aged gonadectomized marmosets mimics the CNS symptoms reported by women taking AIs. Brain tissues were analyzed for estradiol (E2) content and HPC pyramidal cell physiology to investigate effects of Letrozole on the brain. Urine analysis was conducted to determine effects of Letrozole on peripheral levels E2, T, and cortisol. Half the marmosets (females: *n* = 4; males: *n* = 4) were fed 20 µg of Letrozole mixed in approximately 0.3 g pudding (Jell-O) daily for four weeks. The Letrozole dose was determined based on the recommended dose for women

(Bayer et al., 2015; 2.5 mg/day). The remaining marmosets (females: *n* = 3; males: *n* = 5) received pudding without the drug. Group assignment was pseudorandom, with one member of each pair assigned to Letrozole and the other to Vehicle. Both treatment groups were matched based on age and sex. Marmosets were administered the spatial working memory test (i.e. delayed matching-to-position task, DMP) daily for 5 days prior to the start of the drug treatment, and again during the fourth week of treatment. During the final treatment week, experimenters also collected urine for later hormone analysis, video recorded spontaneous behaviors of each marmoset in their home cage, and administered the thermal challenge. The thermal challenge was designed after procedures used in postmenopausal women to induce hot flashes via application of a heating pad to the abdomen (Freedman, 1989; Sievert et al., 2002). After all experimental procedures were complete, the monkeys were euthanized. Electrophysiological recordings were conducted on pyramidal cells in the CA1, and E2 levels were analyzed in several brain regions. All experimenters were blind to group membership.

Treatment administration

Previous animal studies used a route of administration and AI dose that differed from those used by breast cancer patients. In the present study, attempts were made to match route (i.e, oral) and dose to that of humans. The Letrozole dose was determined based on the recommended dose for women (Bayer et al., 2015; 2.5 mg/day). After a one-week baseline period, half the marmosets (females: n = 4; males: n = 4) were fed 20 μg of Letrozole mixed in approximately 0.3 g pudding (Jell-O) daily for four weeks. The remaining marmosets (females: n = 3; males: n = 5) received pudding without the drug. Group assignment was pseudorandom, with one member of each pair assigned to Letrozole and the other to Vehicle. Both treatment groups were matched based on age and sex.

Urine collections and assay

While ovaries are the major source of peripheral E2, previous studies have shown that ovariectomy does not completely abolish E2 levels in the marmoset (Barnett et al, 2006; Lacreuse et al., 2014), suggesting alternate tissues continue producing this hormone. Since AI use should further deplete any E2 synthesis in OVX females and gonadectomized males, peripheral E2 levels was analyzed at the end of the treatment phase. Some studies have also reported increased testosterone (T) following AI use (Taylor et al., 2017), and so T levels were also assessed. Cortisol was also measured as a negative control, as there is little evidence that AIs should influence cortisol synthesis (Bajetta et al., 1999; Rossi et al., 2009). A few minutes before lights on (i.e., 8:30 AM), marmosets entered a stainless steel transport box (34.1 X 20.65 X 30.8 cm) attached to their home cage. They were released once they had urinated, or 15 min had elapsed. Urine was collected using a disposable transfer pipette and placed in a microcentrifuge tube, then spun for 5 min at 14000 rpm. The supernatant was then transferred to a separate tube before being stored at -20ºC. Samples collected were analyzed for these three hormones using enzyme immunoassay (EIA) by the Endocrine BioServices Laboratory at the University of Nebraska at Omaha.

Behavioral Assessments

Elevated levels of E2 are associated with reduced anxiety in women (Maeng & Milad, 2015), and while breast cancer survivors report increased depression and anxiety relative to women with no cancer history (Hansen, Feuerstein, Calvio, & Olsen, 2008), the impact of AI use on these symptoms are poorly understood. To investigate whether daily Letrozole administration impacts anxiety-like behavior in GDX marmosets, spontaneous behaviors of each marmoset pair were recorded daily for 5 days for 10 min alternating between 9-10 AM and 4-5 PM. This ensured that behavioral data was collected at least twice during each time period (AM/PM) for each subject. Treatment groups are represented in each pair, which limits time of day as a potential confound for differences in treatment groups on the observed behaviors. Vigilant and

anxiety-like behaviors (agitated locomotion, inactive alert, scentmark, piloerection), relaxed locomotion (i.e. calm locomotion) and rest (i.e. inactive rest) of each marmoset was later scored by an observer blind to group membership using ODLog (2.7.2, Macropod software 2012). A behavioral ethogram of these six behaviors is presented in Table 1. The duration (in seconds) of each behavior was averaged for AM and PM observations of each subject.

Thermal challenge

Both estrogen deprivation following menopause (De Zambotti et al., 2014; Sievert, 2013) and AI use (Kligman & Yonus, 2010) are associated with hot flashes. Hot flashes result from thermodysregulation as indicated by rapid fluctuations in heat dissipation responses, including skin conductance, skin blood flow and temperature (Freedman, 2014). A common method to induce hot flashes in postmenopausal women is to apply heating pads on their abdomen (Freedman, 1989; Sievert et al., 2002). In the present study, a similar procedure was used. A sitting experimenter held the marmoset on her lap with two hands for 20 minutes. One hand covering the abdomen held a hand warmer that provided the heating source. To prevent overheating, the hand warmer was inserted between two cotton gloves, and had no direct contact with the experimenter's or monkey's skin. For additional protection of the monkey, the hand was covered with a nitrile glove. The hand was kept on the abdomen for a total of 20 min. A second experimenter, sitting about 20 cm from the animal's face, recorded the facial temperature of the marmoset using a thermal imaging camera (FLIR One for iOS) connected to an iPhone 5s (Figure 4A). Thermal imaging has been shown to be an effective method to measure hot flashes in women (Jayasena et al., 2015). The experimenter focused the camera on the marmoset's face using the crosshair of the camera as a guide. The camera provided a video of the face along with a measurement of temperature (in °F) on the crosshair location. The sensitivity of the camera was 0.18° F. One out of every 25 frames was extracted from each video using VLC media player, resulting in approximately 670 frames for each animal (33/min).

An experimenter blind to group assignment visually inspected each frame and removed those for which the spot meter was incorrectly placed (located off the face, or on the eye or mouth). The temperature values obtained from each remaining frame (122 ± 60) were averaged in 1-min bins for each animal. One marmoset was removed from subsequent analyses, as too many frames were removed, leaving 4 min unaccounted for during the 20-min challenge. For every minute of the thermal challenge, each marmoset obtained a change score, which was calculated as the percent change in temperature (°F) from the first min of the challenge (i.e. baseline).

Delayed matching-to-position

Estrogens plays an important role in regulating HPC structure and function in both sexes (Frick, Kim, & Koss, 2018). Aromatase is expressed in the HPC (Hojo et al., 2004), and AIs have been shown to impact HPC-dependent memory in animals (Bailey et al., 2017; Tuscher et al., 2016) and humans (Bayer et al., 2015; Collins et al., 2009). As a first demonstration of the impact of continuous oral AI use in nonhuman primates, we chose a task that is known to involve this structure. The DMP task requires monkeys to discriminate between two locations and displace a token that previously concealed a food reward (Figure 3A). At the beginning of each session, monkeys entered a transport box affixed to their home cage. The front of the box was made of wire mesh, allowing the monkeys to pass their hands and arms to manipulate objects presented in a modified version of the Wisconsin General Testing Apparatus (WGTA; 43.2 x 42.3 x 44.5 cm). The test tray for the DMP task contained 4 food wells (2.5 cm in diameter). All four positions were used and the location of the sample token during each trial was pseudorandomized. Subjects were trained until reliable performance was achieved (8/12 trials over two consecutive days). Only nine marmosets (Vehicle: Males: n = 3; Females: n = 1; Letrozole: Males: n = 3; Females: n = 2) achieved reliable performance following a brief retention delay (1 s) and so were tested (12 trials/day for 5 days) during the baseline and

225 treatment phases. The mean accuracy for each phase (baseline/treatment) was the dependent
226 variable.

227 **Euthanasia and tissue retrieval**

228 Each animal was sedated with ketamine (10 mg/kg, i.m.), then given an intracardial
229 overdose of pentobarbital (50 mg/kg). The brain was removed and the hemispheres were
230 separated. The right hemisphere was immediately placed on dry ice then transferred to a -80 °C
231 freezer for later analyses. The frontal cortex, hypothalamus, and HPC were dissected from the
232 left hemisphere. The HPC was divided in two along the septotemporal axis. The lateral aspect
233 of the HPC was immediately transferred to ice-cold slicing solution (see below). The remaining
234 samples were placed in centrifuge tubes, and stored in a -80 °C freezer.

235 **Electrophysiological recordings**

236 Several lines of research demonstrate the importance of estrogens in regulating the
237 physiology of CA1 neurons. E2 has been shown to decrease the threshold needed to induce
238 LTP (Wong & Moss, 1992). Estrogen deprivation via ovariectomy has been shown to reduce the
239 intrinsic excitability (IE) of these neurons (Wu et al., 2011), yet it is currently unclear whether AIs
240 will have similar effects CA1 physiology. To determine whether E2 synthesis inhibition via
241 Letrozole administration induces comparable changes, similar measures of IE were collected in
242 the present study.

243 Once removed, the HPC was placed in ice-cold slicing solution containing (in mM) 248
244 glycerol, 3 KCL, 1 MgSO4, 2 CaCl2, 1 KH2PO4, 26 NaHCO3, 10 glucose and bubbled with
245 95% O2/5%CO2. Sagittal sections (300 μM) through the HPC were prepared using a VT1000 S
246 vibratome (Leica Biosystems). Slices were incubated at 95°F for 30 minutes in bubbled
247 recording solution containing (in mM) 124 NaCl, 3 KCL, 1 MgSO4, 2 CaCl2, 1 KH2PO4, 26
248 NaHCO3, 10 glucose, after which they were left at room temperature for 30 minutes or up to 8

hours prior to recording. Recording pipettes were pulled from borosilicate glass (4-7 MOhm) using a PC-10 puller (Narishige) and filled with internal solution containing (in mM) 120 K-gluconate, 20 KCL, 0.1 CaCl2, 5 HEPES, 5 EGTA, 3 MgATP, 0.5 Na-GTP, and 10 phosphocreatine. Slices were placed in a recording chamber and perfused with bubbled recording solution throughout the experiments.

A Nikon FN-1 microscope with DIC optics was used to identify pyramidal neurons in the CA1 region. Whole-cell recordings were made with an EPC-10 patch-clamp amplifier and Patchmaster software (HEKA). Resting membrane potential (RMP) and spontaneous spiking activity was measured under current-clamp configuration with zero injected current. To measure excitability, membrane voltage was recorded in response to current injection (-50 pA to +90 pA, 10 pA increments, 500 ms duration). Recordings were discarded if holding current rose above 100 pA or if series resistance rose above 30 MOhm (measured in response to -5mV test pulses in voltage-clamp mode) over the course of a recording.

Data were analyzed offline using custom scripts written in Igor Pro 6 (Wavemetrics). Visualization of data analyses was performed with custom scripts in MATLAB, and visualization of recording traces was performed in Igor Pro. RMP was calculated as the average subthreshold membrane voltage during spontaneous recordings. Current-voltage characteristics were generated, but were not observed to be altered by drug treatment. RMP was derived separately for FI curves and AHP analyses. RMP was determined by finding the average of all points spanning the injection of current, and excluding particular points algorithmically determined to be a part of a spike (defined as those at which the trace's smoothed slope crossed a threshold of 1).

AHP amplitude was derived by finding the minimum value during a window between the peak of the action potential and the return to RMP. This minimum was then subtracted from the RMP, and all points outside of plausible thresholds (-40 mV to -90 mV) were excluded from the

average. Representative recordings from CA1 pyramidal neurons obtained from one vehicle-treated and one Letrozole-treated marmoset are presented in Figure 6. Spontaneous spiking data were collected across the entire recording of 6.5 s by counting crossings of a 0 mV threshold. Similarly, current-evoked firing frequency was found by finding all crossings of a 0 mV threshold within the .5 s window of current injection.

Brain E2 extraction and assay

Aromatase expression occurs in both the hypothalamus and HPC across several species (Vahaba & Remage-Healey, 2015), and so these two regions were selected for analysis of E2 levels. The PFC was chosen as a negative control, as there is currently no evidence that aromatase is expressed in this region. E2 levels were measured in homogenized samples (200 µl in 0.1 M phosphate buffer) of the HPC, frontal cortex, and hypothalamus using an EIA assay (Cayman Chemical) following a combined solid and liquid phase extraction technique described elsewhere (Chao, Schlinger, & Remage-Healey, 2011; Tuscher et al., 2016). Three additional homogenized samples were spiked with E2 with known concentrations (342.4 pg/ml, 856 pg/ml, and 2.14 ng/ml), and another four samples with Letrozole (16 pM, 32 pM, 64 pM, and 94 pM). The extraction process began with three rounds of ether extraction. Once the organic phase was dried, a methanol (MeOH) and dichloromethane (Ch_2Cl_2) solution was carefully released down the sides of the tube before re-evaporating the samples. Dried samples were re-suspended in 0.1 M PB, followed by solid-phase extraction, which consisted of eluting the re-suspended samples through high performance extraction disk cartridges lined with C18 (3M, Eagan, MN, USA) under vacuum pressure (-7 in/Hg). Hydrophilic and hydrophobic (including E2) compounds were eluted using ddH2O (200 µl) and 100% methanol (2x 200 µl), respectively, before air drying in a water bath at 50 °C. A MeOH/Ch_2Cl_2 solution was once again released down the sides of the tube before one final evaporation. Samples were then re-suspended in EIA buffer, and E2 levels in each sample were measured from EIA plates using

299 an Epoch Microplate Spectrophotometer plate reader (Biotek) with a 450 nm filter and Gen5
300 software. An additional three tubes of unextracted standards of equivalent concentrations to the
301 spiked samples (342.4 pg/ml, 856 pg/ml, and 2.14 ng/ml) were included to determine the
302 effectiveness of the extraction protocol in reducing assay interference. The average recovery
303 rate of the extraction efficiency tubes was 98% (SD = 22.85%) indicating high correlation
304 between expected and obtained values. In addition, the E2 levels obtained in the samples
305 spiked with Letrozole showed no evidence of cross reactivity with the ELISA antibody.

306 **Statistical Analysis**

307 To maintain blindness to treatment assignment, codes were assigned to both groups to
308 run statistical analyses, and were decoded once all analyses were complete. All statistical
309 analyses were conducted using the *Statistical Program for the Social Sciences* software (IBM,
310 Chicago, IL), with type I error rate set at $\alpha = .05$, and results are expressed as mean (± SEM) in
311 Figures 1-5. Peripheral and central hormone levels (Figures 1-2) were analyzed using two-way
312 analysis of variance (ANOVA) with treatment and sex as factors. The same analysis was run on
313 spontaneous behaviors (Table 2), with follow-up ANOVAs within each sex. Given the advanced
314 age of some of the marmosets, some were unable to obtain criterion performance on the DMP
315 task, and so sex was not included as a factor. Since performance was expected to decrease for
316 the Letrozole, but not the Vehicle group as treatment progressed, change from baseline to the
317 final treatment was analyzed separately within each group using a paired-samples t-test (Figure
318 3B). Percent change in temperature from baseline during the thermal challenge was analyzed
319 using a three-way ANOVA, with treatment and sex as between-subjects factors, and time as a
320 within-subjects factor (Figure 4B-C). Follow-up two-way ANOVAs were conducted within each
321 sex, with time and treatment as factors. Independent-samples t-tests were used to analyze
322 treatment effects on passive properties of IE of CA1 neurons (Figure 5B-D). To examine the

firing frequency at increasing current (-50-+90 pA; Figure 5A), a two-way ANOVA with follow-up independent-samples t-tests were conducted.

In addition to investigating group differences on E2 levels and each outcome measure, selective relations were also examined. Since E2 synthesis inhibition in the HPC has been shown to impair HPC-dependent memory, it was expected that HPC-E2 would correlate positively with DMP task performance and excitability of CA1 neurons. It was also predicted hypothalamic E2 would correlate negatively with the degree of facial temperature change during the thermal challenge, given that thermosensitive neurons responsible for maintaining body temperature are located in the preoptic area (Rance et al., 2013).

Results

Letrozole lowers peripheral E2 levels

To confirm that 4 weeks of Letrozole decreases peripheral levels of E2 in each sex, comparisons were run on urinary levels of the hormone (Figure 1A). Additional analyses were run on T (Figure 1B) and cortisol (Figure 1C) to determine whether these related hormones are also influenced by the treatment. A main effect of treatment was observed for E2 ($F(1,12) = 24.69$, $p = .0003$, $\eta^2 = .67$; Figure 1A), with lower levels observed in the Letrozole-treated group (0.03 µg/mg Cr ± 0.004) compared to the Vehicle group (0.33 µg/mg Cr ± 0.07; Hedge's g = -2.17). No effect of sex ($F(1,12) = 1.57$, $p = .24$, $\eta^2 = .12$) or interaction ($F(1,12) = 1.43$, $p = .25$, $\eta^2 = .11$) was observed. Similar levels of T were observed across treatment group and sex, with no significant main effect or interaction (sex: $F(1,12) = 1.14$, $p = .307$, $\eta^2 = .09$; drug: $F(1,12) = 0.22$, $p = .645$, $\eta^2 = .02$; interaction: $F(1,12) = 1.82$, $p = .20$, $\eta^2 = .13$; Figure 1B). A main effect of sex was observed for cortisol ($F(1,12) = 5.71$, $p = .034$, $\eta^2 = .32$), with higher levels observed in females (35.22 µg/mg Cr ± 12.85) than males (14.33 µg/mg Cr ± 1.90, Hedge's g = 1.11). No effect of treatment ($F(1,12) = 0.23$, $p = .64$, $\eta^2 = .02$, Figure 1C) or interaction ($F(1,12) = 0.13$, $p = .73$, $\eta^2 = .01$) was observed for cortisol.

Letrozole increases HPC-E2 levels

E2 levels in the HPC, hypothalamus, and frontal cortex were analyzed next. Results revealed a main effect of treatment on E2 levels in the HPC ($F(1,10) = 6.33$, $p = .031$, $\eta^2 = .39$; Figure 2A), with higher levels in the Letrozole-treated (1471.32 pg/g ± 311.97) than in the control animals (522.67 pg/g ± 300.76; Hedge's g = 1.12). No effect of sex ($F(1,10) = 0.93$, $p = .36$, $\eta^2 = .09$) or interaction ($F(1,10) = 2.86$, $p = .12$, $\eta^2 = .22$) was observed. No main effects or interactions were observed for the hypothalamus (sex: $F(1,9) = 0.84$, $p = .38$, $\eta^2 = .09$; treatment: $F(1,9) = 1.00$, $p = .36$, $\eta^2 = .17$; interaction: $F(1,9) = 1.78$, $p = .22$, $\eta^2 = .17$; Figure 2B). or frontal cortex (treatment: $F(1,11) = 1.13$, $p = .307$, $\eta^2 = .09$; sex: $F(1,11) = 0.10$, $p = .76$, $\eta^2 = .01$; interaction: $F(1,11) = 0.21$, $p = .66$, $\eta^2 = .02$; Figure 2C).

Letrozole decreases some anxiety-like behaviors

To determine whether oral Letrozole given to marmosets mimics mood changes reported by women taking AIs, spontaneous behaviors were compared across treatment and sex. A significant main effect of treatment was observed for time spent in agitated locomotion in the AM ($F(1, 12) = 5.12$, $p = .043$, partial $\eta^2 = .30$), with longer duration observed in the Letrozole group (6.16 s ± 2.02) than the Vehicle group (0.86 s ± 0.41; Hedge's g = 1.28). An interaction was observed for time spent in inactive alert in the afternoon ($F(1, 12) = 10.90$, $p = .006$, partial $\eta^2 = .48$). Follow-up comparisons revealed more time spent in inactive alert for males treated with Letrozole than controls ($F(1, 7) = 6.03$, $p = .044$, partial $\eta^2 = .46$; Letrozole = 375.22 s ± 56.51; Vehicle = 180.92 ± 54.36, Hedge's g = 1.65), whereas a marginally significant effect was found in females ($F(1, 5) = 4.81$, $p = .08$, partial $\eta^2 = .49$; Letrozole = 166.82 s ± 55.79; Vehicle = 395.48 ± 96.46, Hedge's g = -1.68). No other comparisons were significant. Table 2 presents the mean duration of each behavior for the Vehicle and Letrozole-treated group in the morning and afternoon.

Letrozole impairs HPC-dependent cognition

A statistically significant decrease in performance was observed in the Letrozole-treated group ($t(4) = 3.66$, $p = .022$, Hedge's g = 1.16; Performance change: Males = 4.42% ± 2.18; Females = 10% ± 0), whereas no change was observed in the vehicle-treated group ($t(3) = 0.40$, $p = .71$, Hedge's g = 0.34, Figure 3B; Males = -0.26% ± 15.68; Females = 12.27% ± 0, Figure 2B).

Letrozole increases facial temperature during thermal challenge in females

Letrozole-induced change in facial temperature during a thermal challenge was investigated to determine whether marmosets in the Letrozole group experience thermodysregulation, mimicking hot flashes reported by women taking AIs. A main effect of sex ($F(1, 4) = 8.81$, $p = .041$, $\eta^2 = .69$) and time ($F(19, 76) = 21.69$, $p = .000$, partial $\eta^2 = .84$), a two-way interaction between treatment and sex ($F(1, 4) = 15.26$, $p = .017$, $\eta^2 = .79$), treatment and time ($F(19, 76) = 2.62$, $p = .002$, partial $\eta^2 = .39$), and sex and time ($F(19, 76) = 2.64$, $p = .001$, partial $\eta^2 = .40$), and a 3-way interaction between sex, treatment, and time ($F(19, 76) = 2.72$, $p = .001$, partial $\eta^2 = .40$). A follow-up two-way ANOVA conducted within each sex revealed a main effect of treatment ($F(1, 3) = 17.10$, $p = .026$, $\eta^2 = .85$), time ($F(19, 57) = 15.09$, $p = .000$, partial $\eta^2 = .83$), and an interaction between treatment and time ($F(19, 57) = 3.37$, $p = .000$, partial $\eta^2 = .53$) among females (Figure 4B), with greater change in facial temperature in the Letrozole group (Vehicle = 0.54 %change in °F ± 0.05 Letrozole = 4.45 %change in °F ± 1.46; Hedge's g = 1.55). A main effect of time was observed in males ($F(19, 19) = 6.96$, $p = .000$, partial $\eta^2 = .87$), but all other effects were not significant (treatment: $F(1, 1) = 2.80$, $p = .343$, $\eta^2 = .74$; interaction: $F(19, 19) = 1.51$, $p = .188$, partial $\eta^2 = .60$; Figure 4C). Temperature change was similar across the two treatment groups in the males (Vehicle = 2.20 %change in °F ± 1.75 Letrozole = 1.94 %change in °F ± 0.48; Hedge's g = -0.09).

Letrozole reduces IE of CA1 neurons

To observe whether oral Letrozole mimics changes in HPC activity known to occur following ovarian hormone deprivation, intrinsic activity of CA1 neurons were compared across treatment groups. FI curves were compared and revealed a significant interaction between treatment and injected current ($F(4,100) = 2.95$, $p = .024$, $\eta^2 = .11$), with reduced firing frequency in Letrozole-treated cells following currents of 20 pA ($t(27) = -2.69$, $p = .012$, Letrozole = 1.47 Hz ± 0.54, Vehicle = 4.46 Hz ± 1.00,Hedge's g = -1.00) and 30 pA ($t(27) = -2.25$, $p = .013$, Letrozole = 2.43 Hz ± 0.62, Vehicle = 5.57 Hz ± 1.03, Hedge's g = -0.98; Figure 6A). Significant treatment effects were observed on passive measures, with CA1 neurons from the Letrozole-treated group demonstrating higher AHP amplitude ($t(22) = 2.25$, $p = .035$, Hedge's g = 0.92, Figure 6B, Letrozole = 0.02 Hz ± 0.002, Vehicle = 0.01 Hz ± 0.001), lower resting membrane potential ($t(22) = -2.18$, $p = .041$, Hedge's g = 0.89, Figure 6C, Letrozole = -0.06 mV ± 0.002, Vehicle = -0.05 mV ± 0.001), and lower spontaneous spiking ($t(22) = -2.10$, $p = .048$, Hedge's g = 0.86, Figure 6D; Letrozole = 1.43 mV ± 0.71, Vehicle = 1.67 mV ± 0.65) relative to neurons from the Vehicle group.

Correlations between study measures

The relation between E2 levels (peripheral and central) were analyzed for all primary outcome measures. Among the Letrozole-treated marmosets, hypothalamic E2 was related to facial temperature change at four of the 20 time points ($r(4) = .94$-$.98$, $p < .006$, Bonferroni corrected), whereas no associations were observed for control animals. Peripheral levels of E2 did not correlate with facial temperature change in either treatment group.

Peripheral E2 levels were not associated with performance on the DMP ($r(7) = .35$, $p = .360$) or measures of extrinsic activity of CA1 neurons ($r(6) = .44$-$.59$, $p = .213$-$.381$). A large, non-significant negative correlation was found between HPC-E2 levels and the drop in DMP performance in the Letrozole group (r(3) = -.87, p = .058, see Figure 3B). No such correlation was observed in the Vehicle-treated group ($r(2) = .38$, $p = .623$). The relation between CA1

activity (AHP, RMP, and spontaneous spiking) and neuroestradiol was also not significant ($r(6) = .08\text{-}.86$, $p = .27\text{-}.89$).

Discussion

Four weeks of an oral AI (Letrozole; 20 µg/day) has adverse effects on multiple behavioral, neuronal and physiological outcomes in GDX marmosets over 5 years old. Reduced performance on the DMP task from baseline to the final treatment week was observed in the Letrozole-treated marmosets, whereas no change was observed in controls (Figure 2B), suggesting that Letrozole reduces spatial working memory ability. This is consistent with previous studies demonstrating impaired memory following aromatase inhibition in the HPC in rats (Tuscher et al., 2016), songbirds (Bailey et al., 2017, but see Taylor et al., 2017), and humans (Bayer et al., 2005; Collins et al., 2009, but see Lee et al., 2016).

Letrozole also reduced the intrinsic excitability (IE) of pyramidal neurons in the CA1 on multiple measures in both sexes. Relative to controls, neurons from the Letrozole-treated animals demonstrated reduced responsiveness to injected current, and lower spontaneous activity. These properties together reflect a compromised state of IE, since they influence how effective synaptic inputs translate to action potentials. The reduced IE of CA1 neurons is consistent with results reported in rats following short- (2 months) or long-term (5 months) ovarian hormone deprivation (Wu, Adelman, & Maylie, 2011; via OVX). In that study, long-term OVX resulted in reduced neuronal responsiveness to injected current, and steeper AHP slope. Taken together, these results suggest that 4 weeks of Letrozole treatment mimics the detrimental effects of long-term ovarian hormone deprivation on IE and spontaneous activity. While E2 is known to enhance excitatory synaptic transmission in the HPC (Smejkalova & Woolley, 2010; Wong & Moss, 1992; Wu et al., 2011), peripheral and HPC-E2 levels were not correlated with IE, suggesting that Letrozole impacts HPC physiology via other mechanisms. One advantage of the present study over previous research is that neurons from both males

447 and females were included. Since the data are consistent across both sexes, these results
448 indicate that Letrozole has detrimental effects on the IE of CA1 neurons irrespective of sex.

449 The increase in facial temperature in response to a thermal challenge was higher in
450 Letrozole-treated monkeys than controls, but in female marmosets only. This indicates that
451 Letrozole-treated females were less able to regulate their body temperature than vehicle-treated
452 females. E2 is known to decrease core body temperature in women (Brooks et al., 1997;
453 Tankersley et al., 1992), female rodents (e.g. Dacks & Rance, 2010) and female marmosets
454 (Gervais et al., 2016), and AI treatment triggers hot flashes in breast cancer patients (Kligman &
455 Younus, 2010). Both endogenous E2 levels (Baker et al., 2015) and estrogen therapy
456 (Freedman & Blacker, 2002) are associated with fewer hot flashes. Circulating E2 levels were
457 not associated with change in facial temperature in either group in the present study, but
458 hypothalamic E2 levels correlated positively with facial temperature change within the Letrozole-
459 treated marmosets. As the hypothalamus, and in particular the preoptic area (POA), contains
460 thermosensitive neurons that play a role in thermoregulation (as reviewed by Rance et al.,
461 2013), it is possible that aromatase inhibition impairs thermoregulation via mechanisms that
462 involve E2 in this structure. No group differences in hypothalamic E2 levels were observed, but
463 the analyzed tissue included all sub-regions of the hypothalamus, and so any effect on the POA
464 might have been masked. While the results of the present suggest that aromatase inhibition
465 compromises thermoregulation in a sex-specific manner, other known sex differences can also
466 explain the observed findings, including differences in the POA (Ayoub, Greenough, & Juraska,
467 1983), body physiology, anthropometric characteristics, and body composition (as reviewed by
468 Kaciuba-Uscilko & Gruza, 2001). In addition, we cannot rule out that the observed sex
469 difference resulted from an interaction between the potential stress associated with the thermal
470 challenge and sex. Future studies are needed to better understand sex differences in
471 thermoregulation and the contribution of neuroestradiol in regulating thermosensitive neurons.

Some vigilant behaviors were increased by Letrozole treatment, although time of day appears to have influenced the pattern of results. In the morning, Letrozole-treated marmosets spent more time in agitated locomotion, a sensitive marker for anxiety (Bowell, 2010). Consistent with this, males taking Letrozole spent more time in inactive alert in the afternoon relative to control males, indicative of heightened vigilance, a behavioral marker similar to trait anxiety in humans (Shiba et al., 2014). Letrozole had no effect on this behavior in females. Thus, greater inactive alert and agitated locomotion likely reflect greater anxiety. There are inconsistencies with regards to the effects of aromatase inhibition on anxiety in rodents, with one study showing impaired fear extinction (i.e. greater fear maintenance) in male rats following acute inhibition (Graham & Milad, 2014), and others reporting no effect of sustained inhibition on open field activity in OVX/castrated rats (Kokras et al., 2018), and no difference between ArKO and wild type mice on the open field and elevated plus maze (Dalla et al., 2005). While the results of the current study suggest that daily oral AI can have anxiogenic properties, more research in this area is needed before firm conclusions can be drawn.

As predicted, peripheral levels of E2 were reduced by Letrozole treatment, but a region-specific effect was observed in the brain, with higher E2 levels detected in the HPC of Letrozole-treated tissue, and no effect in the PFC and hypothalamus. Letrozole clearly crosses the blood brain barrier as demonstrated in rats (Dave, Gudelsky, & Desai, 2013), with exposure to the brain being dose-dependent. It is possible that the Letrozole dose was too low to effectively suppress aromatase activity in the brain, whereas it did so at the periphery. In fact, our data are consistent with a ‘compensation’ hypothesis, suggesting that elevated HPC-E2 levels result from increased aromatase expression and/or activity in response to lower peripheral level of E2. In support of this interpretation, increased aromatase expression is observed following OVX in rhesus monkeys (Higaki et al., 2012). Further, data in zebra finches show that continuous administration of oral AI leads to increased expression of aromatase protein, while elevated

peripheral levels of E2 downregulates aromatase in the HPC, but not other brain areas, including the POA (Saldanha et al., 2000). Such region-specific patterns of aromatase expression are consistent with our data, which show differential responses of the HPC vs. hypothalamus and frontal cortex Studies using direct administration of AIs within the HPC show reduced E2 synthesis in the HPC, both in female mice (Tuscher et al., 2016) and male songbirds (Bailey et al., 2017). Similarly, in vitro studies of hippocampal neurons demonstrate reduced E2 synthesis following Letrozole administration through a mechanism of phosphorylation that inactivates aromatase without decreasing aromatase expression (Fester et al, 2016). Similarly, E2 levels may be altered centrally due to alterations in catabolism or inactivation of estrogens by enzymes such as suppression of 2-hydroxylase (Zhu & Conney, 1998; Osawa et al., 1993). The mechanisms underlying differential effects of Letrozole according to the mode (oral or central) of administration remain to be determined.

As mentioned above, the dose of administration is important for the extent of brain exposure. There is also evidence that the duration of administration may be crucial to understanding Letrozole mechanisms. ER-responsive tumors can often escape inhibition with prolonged Letrozole treatment (Ma et al., 2015). There is also evidence for differential effects of Letrozole on depressive-like behavior in female rats depending on whether it is administered acutely (3 injections in 24 h) or continuously (7 days; Kokras et al, 2014). Because AIs are administered both orally, at low dose and continuously in women, one prediction from our study is that they induce elevated HPC E2 levels, an intriguing possibility that will require empirical validation.

The present results suggest that *higher* HPC-E2 levels are related to greater performance deficits on the DMP task in the Letrozole group, which is in contradiction with studies using intracerebral administration of AIs, finding impaired spatial learning and memory Tuscher et al., 2016; Bailey et al., 2017) following *reduced* E2 levels in the HPC. While no

correlation was observed, elevated HPC-E2 levels might also lead to reduced IE of CA1 neurons. As predicted by the compensation hypothesis, prolonged use of AIs may reduce the functionality of the HPC by increasing aromatase expression/activity, resulting in elevated HPC-E2 levels, impaired spatial memory, and compromised IE of CA1 neurons. Prolonged use of Letrozole might also lead to the observed findings through escaped inhibition. Future studies are needed to address these important questions.

This was the first study using a nonhuman primate to examine the effects of Letrozole on brain and behavior. We used a regimen that mimics AI use in humans, examined a large number of behavioral measures, spanning cognition to anxiety to thermoregulation and analyzed sex differences. At the brain level, we measured both E2 content in several brain regions and HPC neurons physiology. Such a comprehensive approach in an animal model phylogenetically close to humans is unique and a great strength of the study. There were also limitations associated with the use of nonhuman primates. The relatively small sample, the broad age range, and the use of a single cognitive task limit the conclusions that can be drawn from our results.

Nevertheless, we demonstrated that 4 weeks of oral administration of the AI Letrozole at a dose comparable to what is used by women effectively reduces peripheral levels of E2 but leads to a compensatory increase in E2 levels in the HPC. Thermoregulation, HPC neural activity and spatial working memory were all compromised by treatment. These results in a nonhuman primate corroborate many of the symptoms reported by women taking AIs (Bender et al., 2007; Rand et al., 2011) and reveal detrimental effects of these treatments on the brain, in part through elevated HPC-E2 levels. Future studies are needed to elucidate the precise mechanisms by which AIs compromise the CNS.

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748 **Figure legend**

749 **Figure 1**. **Four weeks of Letrozole treatment (20 µg/day, p.o.) lowers circulating levels of**
750 **E2, but has no effect on testosterone or cortisol.** Urinary hormone levels were collected from
751 a mixed-sex sample of marmosets, who received either Letrozole (Males: *n* = 4; Females: *n* = 3)
752 or Vehicle (Males: *n* = 4; Females: *n* = 4). (**A**) 17β-estradiol levels were significantly lower in the
753 Letrozole-treated than the control group (**p* < .001). (**B**) Free testosterone and (**C**) cortisol levels
754 were not different across treatment groups, but were higher in females than males (**p* < .05).
755 Mean values obtained in each group are presented in µg/mg Cr. Error bars = SEM.

756 **Figure 2. Region-specific increase in 17β-estradiol (E2) levels following 4 weeks of**
757 **Letrozole (20 µg/day, p.o.)**. Brain regions analyzed were (**A**) the hippocampus, (**B**)
758 hypothalamus, and (**C**) frontal cortex. Letrozole (Males: *n* = 4; Females: *n* = 3) was associated
759 with higher E2 levels in the hippocampus relative to the control group (Males: *n* = 4; Females: *n*
760 = 4; **p* < .05). Remaining regions were unaffected by drug treatment. No effect of sex on E2
761 levels of any region. Mean values obtained in each group are presented in pg/g. Error bars =
762 SEM. Dashed horizontal lines indicate the average background E2 levels obtained by EIA for
763 assay blanks.

764 **Figure 3. Letrozole (20 µg/day, p.o.) impairs spatial working memory, possibly by 17β-**
765 **estradiol in the hippocampus**. (**A**) Procedure for a delayed matching-to-sample (DMP) test
766 trial includes the sample phase, when a monkey is presented with a red token placed over one
767 of the 4 wells, and is allowed to displace the token to obtain a reward (dehydrated mini
768 marshmallow). The test tray is then concealed from view for 1 s, during which time the reward is
769 replaced and the token is re-positioned over the sample location along with an identical token
770 placed over a different well. During the test phase, the monkey must displace the token located
771 in the same position to obtain the reward. Performance was averaged across trials given during
772 a baseline phase, and after 4 weeks of drug treatment. Performance, which was measured as

773 percentage of trials correct, was compared across phases within each experimental group
774 (Vehicle: *n* = 4; Letrozole: *n* = 5). (**B**) Mean accuracy of each group during the baseline and
775 treatment week. Reduced performance was observed in the Letrozole group (*p < .05; *n* = 5),
776 while performance in the Vehicle group (*n* = 4) did not change. Error bars = SEM. (**C**) A larger
777 reduction in performance is associated with higher E2 levels in the hippocampus within the
778 Letrozole group (*r* = -.87, *p* = .058).

779 **Figure 4. Letrozole (20 µg/day, p.o.) exerts sex-specific reduction in thermoregulation**
780 **during thermal challenge.** (**A**) Facial temperature was measured via thermal camera during a
781 20-min thermal challenge. Representative images obtained 5 min and 20 min into the challenge
782 are shown, along with the temperature reading. Percent change in temperature (°F) from the
783 first min of the thermal challenge was plotted over time for females (**B**; Vehicle: *n* = 2; Letrozole:
784 *n* = 4) and males (**C;** Vehicle: *n* = 5, Letrozole: *n* = 4). Letrozole treatment resulted in greater
785 elevation in temperature across time for females only (*p* < .001). Error bars = SEM.

786 **Figure 5. IE of CA1 pyramidal neurons is attenuated by 4 weeks of Letrozole treatment**
787 **(20 µg/day, p.o.; *p < .05 on all 4 measures)**. This is observed across 4 measures. (**A**) Firing
788 frequency (Hz) is attenuated as a function of injected current from Letrozole-treated cells (*n* =
789 14) relative to Vehicle (*n* = 15). This shift in excitability was also related to changes in
790 spontaneous activity by Letrozole treatment, as indicated by (**B**) greater after hyperpolarization
791 amplitude (AHP; mV) (**C**) lower resting membrane potential (RMP; mV), and (**D**) lower
792 spontaneous spike rate (Hz). Error bars = SEM.

793 **Figure 6. Representative recordings from CA1 pyramidal cells from animals following**
794 **vehicle treatment (A) and Letrozole treatment (B)**. Left: voltage traces during 500-ms current
795 steps (grey, -50 pA; red, rheobase; black, twice rheobase). Right: single spontaneous action
796 potentials showing differences in AHP amplitude.

Table legend

Table 1. Marmoset behavioral ethogram

Table 2. Average duration (in seconds) of each behavior during 10-min session recorded during final treatment week

801 Table 1.

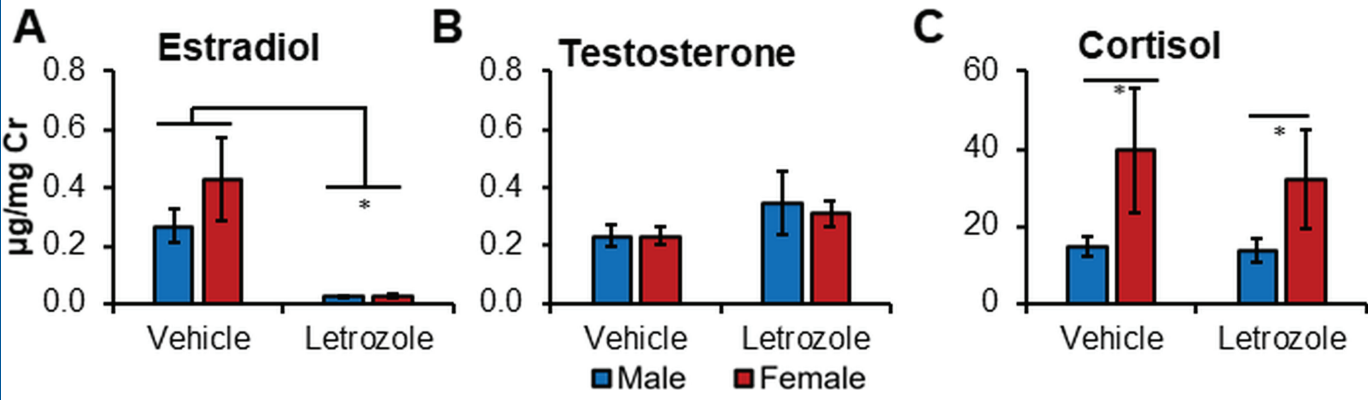
Behavior	Definition
Agitated locomotion	Rapidly moving between locations with an exaggerated gait. Tail extended or arched
Inactive alert	Stationary behavior involving continuous head movements while scanning environment
Scentmark	Rubbing sternal (tummy) or anogenital area (more commonly) over surface/substrate
Piloerection	Puffing of the body hair
Calm locomotion	Movement between locations with relaxed gait
Inactive rest	Stationary behaviour with relaxed facial expression and minimal scanning. Tail can be curled under body, and eyes may be closed

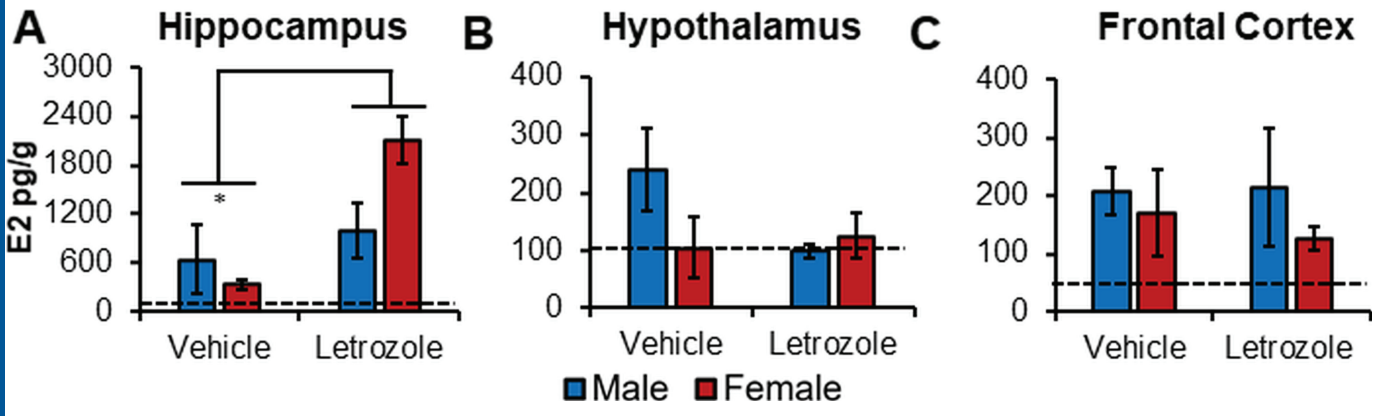
802

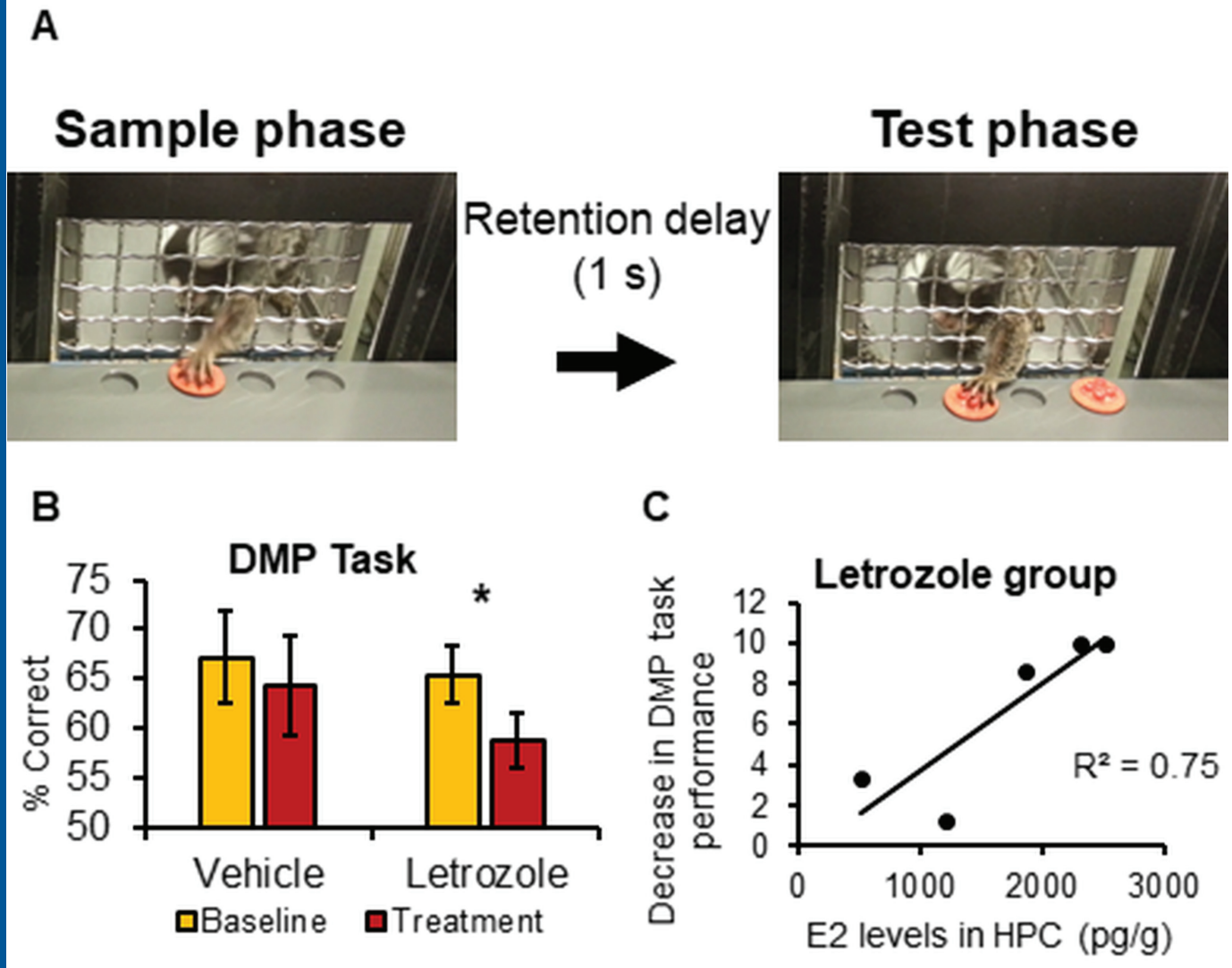
803 Table 2.

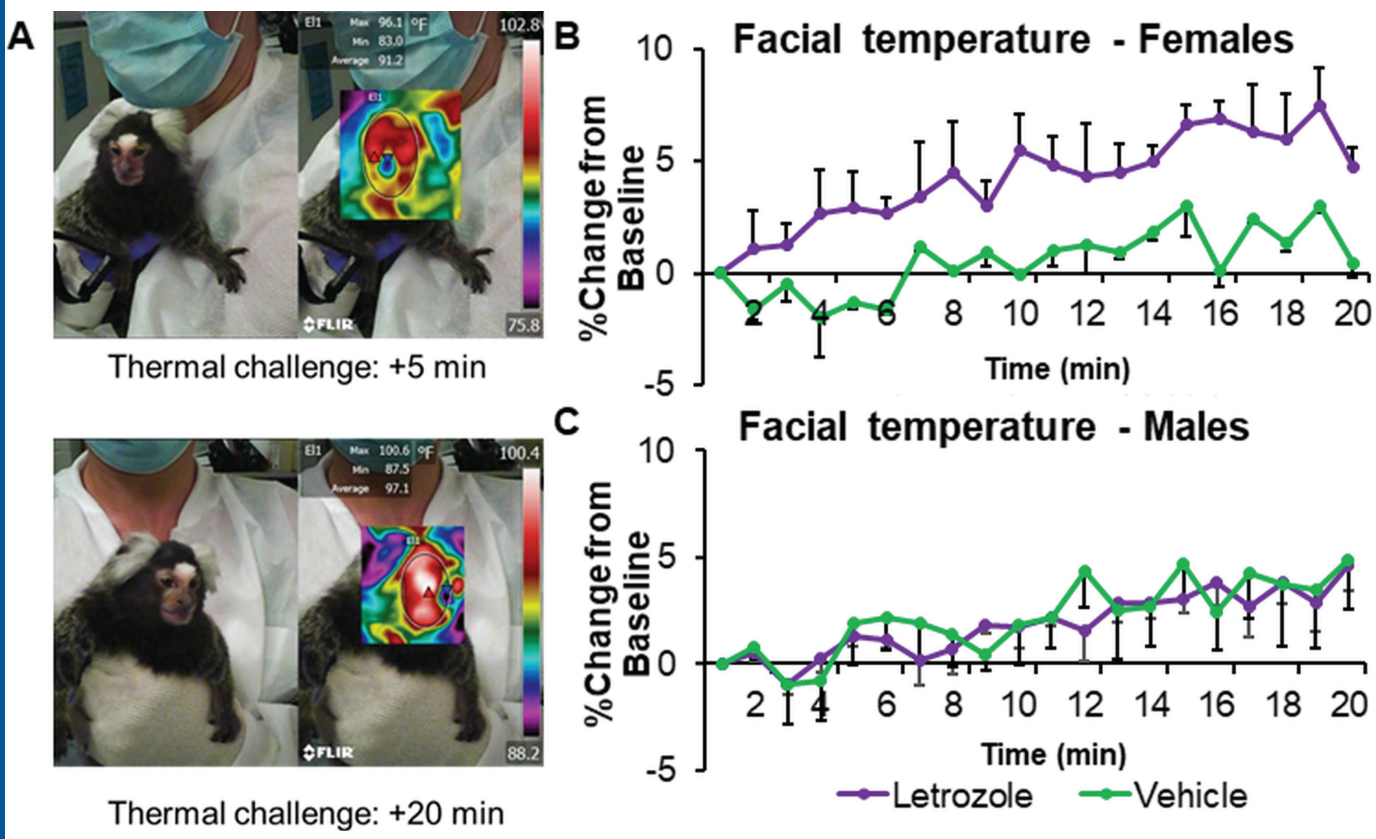
	AM		PM	
Behavior	Vehicle	Letrozole	Vehicle	Letrozole
Agitated locomotion*	0.86 ± 0.41	6.16 ± 2.02	2.57 ± 0.43	0.58 ± 0.20
Inactive alert*	220.54 ± 51.89	291.98 ± 62.58	261.38 ± 59.95	271.02 ± 53.87
Gouge	5.28 ± 3.66	0.21 ± 0.43	0.58 ± 0.37	0.79 ± 0.79
Scentmark	0.78 ± 0.54	0.13 ± 0.13	0	0
Piloerection	0	0.08 ± 0.08	0.40 ± 0.40	0
Calm locomotion	16.88 ± 4.25	11.11 ± 3.01	11.16 ± 2.86	13.88 ± 4.19
Inactive rest	65.06 ± 45.67	16.37 ± 10.78	13.18 ± 6.46	47.06 ± 16.04

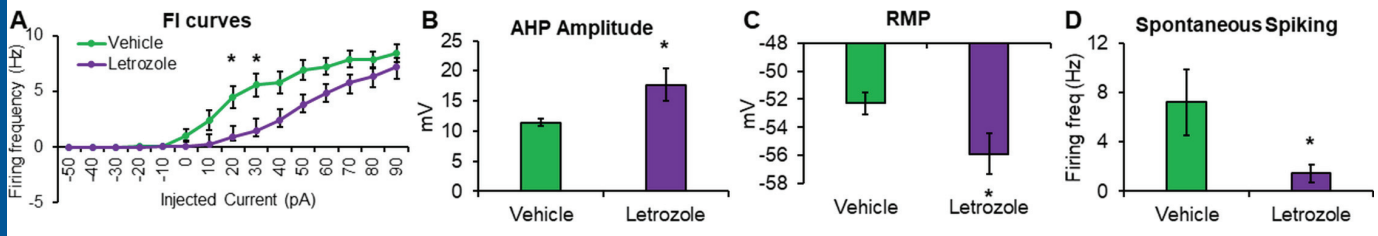
804 *Note*: Values represent mean ± standard error of the mean. Letrozole-treated marmosets spent more time in agitated locomotion
805 than those treated with Vehicle in the AM (*p = .043). Males treated with Letrotzole spent more time in inactive alert than Vehicle-
806 treated males in the PM (*p = .044).











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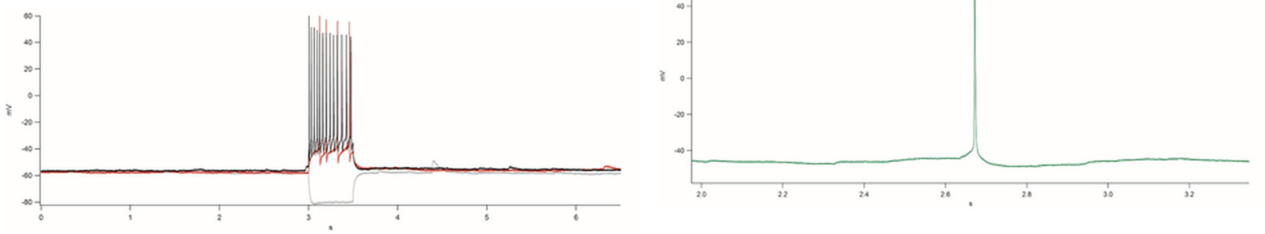

B