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Infusion of Gabrα6 siRNA into the trigeminal ganglia increased the myogenic orofacial nociceptive response of ovariectomized rats treated with 17β-estradiol

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Abstract

High levels of 17β-estradiol (E2) have been found to reduce inflammatory temporomandibular joint (TMJ) pain. A search for genes effected by a high concentration of estradiol showed an increase in $GABA_A$ receptor subunit α6 (Gabrα6) in the trigeminal ganglia (TG). Blockade of Gabrα6 expression in the TG increases masseter muscle nociception in male rats, but the relationship between estradiol's effect on nociception and Gabrα6 expression remains unclear in females. To address this knowledge gap we hypothesized that reducing Gabrα6 expression in the TG will increase the orofacial nociceptive response of ovariectomized female rats treated with estradiol. To administer hormone osmotic pumps were placed in rats that dispensed a low diestrus plasma concentration of 17β-estradiol, in addition, 17β-estradiol was injected to produce a high proestrus plasma concentration of estradiol. A ligature was then placed around the masseter tendon to induce a nociceptive response; a model for TMJ muscle pain. Gabrα6 siRNA was later infused into the TG and the nociceptive response was measured using von Frey filaments and a meal duration assay. $GABA_A$ receptor expression was measured in the TG and trigeminal nucleus caudalis and upper cervical region ($Vc\text{-}C_1$). Ligature significantly increased the nociceptive response but a high proestrus concentration of 17β-estradiol attenuated this response. Gabrα6 siRNA infusion decreased Gabrα6 expression in the TG and $Vc\text{-}C_1$ but increased the nociceptive response after 17β-estradiol treatment. The results suggest estradiol decreased the orofacial nociceptive response, in part, by causing an increase in Gabrα6 expression.

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-Treating with a high physiological concentration of estradiol decreased the nociceptive response and increased Gabrα6 expression
-Infusing Gabrα6 siRNA into the trigeminal ganglia reduced Gabrα6 expression in the trigeminal ganglia and trigeminal nucleus caudalis
-Reducing Gabrα6 expression partially reversed the decreased nociceptive response normally observed after treating with a high physiological concentration of estradiol

Introduction

Estrogens have been shown to effect sensitivity in the human female temporomandibular joint (TMJ) (LeResche et al., 2003; Smith et al., 2006). Moreover, different concentrations of estradiol during the menstral cycle affect the orofacial pain response (Yu et al.). To determine how estradiol could potentially alter orofacial sensitivity, gene expression in the TMJ, trigeminal ganglia (TG) and trigeminal subnucleus caudalis/upper cervical cord region (Vc/C_{1-2}) was analyzed with gene arrays after treating rats with physiological doses of 17 β-estradiol (Puri et al., 2011). A subunit of the $GABA_A$ receptor, Gabrα6, was affected by 17 β-estradiol in the TG and Vc-C_1 (Puri et al., 2011). In addition, a later study found Gabrα6 positive neurons located in the TG projected to the temporomandibular joint (TMJ) (Puri et al., 2012). $GABA_A$ receptors are expressed in sensory neurons of the face and head and have been shown to have a role in modulating orofacial pain (Almond et al., 1996; Anderson et al., 2009; Cai et al., 2001; Cairns et al., 1999; Ginestal and Matute, 1993; Hayasaki et al., 2006; Kondo et al., 1995; Kramer and Bellinger, 2013; Viggiano et al., 2004; Vit et al., 2009). Previous studies demonstrated that glutamate decarboxylase (GAD) 65 within the TG results in GABA production (Hayasaki et al., 2006). This GABA can bind the $GABA_A$ receptor to induce an inhibiting chloride current (Hayasaki et al., 2006), whereas the application of a $GABA_A$ receptor antagonist to the TG reversed neuronal inhibition that results from enhancing GABA expression (Vit et al., 2009). Inhibition of Gabrα6 expression will increase cellular activation of TG neurons and increase the orofacial nociceptive response (Kramer and Bellinger, 2013; Puri et al., 2012). Increased in Gabrα6 expression (Puri et al., 2011) was shown to be concurrent with a decrease in the nociceptive response after administering a proestrus dose of estradiol (Kramer and Bellinger, 2009). While Gabrα6 expression has been shown to increase with a high concentration of 17β-estradiol (i.e., proestrus concentration) (Puri et al., 2011) the role of the $GABA_A$ receptor in orofacial nociception is unclear in females.

To address the question on the role of the $GABA_A$ receptor in orofacial nociception in females we used a myogenic nociceptive model. In this model a ligature was placed on the tendon attachment of the anterior superficial portion of the rat masseter muscle (TASM) (Guo et al., 2010; Kramer and Bellinger, 2013). This ligature model has been shown to induce inflammation and biochemical mediators that act on adjacent neurons and surrounding tissues to produce a nociceptive response (Guo et al., 2010), similar to the tenomyositis and muscle pain observed in a human temporomandibular joint disorder (TMD) (Guo et al., 2010). Orofacial pain, such as TMJ pain, can have a myogenic component (Bailey et al., 1977; Franks, 1965; Helkimo et al., 1979) and since TMD often involves muscle (Stohler, 1999) and muscle tendon pain (DuPont and Brown, 2009); with males reporting less facial muscle pain than females (Zubieta et al., 2002) the TASM model was representative of this type of TMD.

Based on the work cited above, we hypothesized that Gabrα6 has a role in decreasing the TASM nociceptive response after estradiol administration. To directly test this hypothesis we knocked down Gabrα6 expression in the TG and measured the nociceptive response of ovariectomized (OVX) female rats treated with a proestrus dose of estradiol.

Materials and Methods

In Experiment 1, female rats were treated with estradiol and the nociceptive response was measured pre and post ligature. In Experiment 2 the TG of estradiol treated female rats were infused with Gabrα6 small interfering RNA (siRNA) into the TG and then the nociceptive response was measured. Gaba receptor subunit expression was also measured in the TG and Vc-C_1 in Experiment 2.

Animal care and welfare (Experiment 1 and 2)

All animal experiments were approved by the Texas A&M University Baylor College of Dentistry Institutional Animal Care and Use Committee in accordance with the guidelines of the USDA, NIH Office of Laboratory Animal Welfare and National Research Council's "Guide for Care and Use of Laboratory Animals". Female Sprague-Dawley rats (250-300g) were purchased from Harlan Industries, Houston, TX. Upon arrival the animals were housed individually in a temperature-controlled room (23°C) and kept on a 14:10 light/dark cycle with lights on at 06:00 hours. Rats were also gentled by daily handling. The rats were given chow (Harlan Industries) and water ad libitum.

Estrogen supplementation (Experiment 1 and 2)

Rats were anesthetized with ketamine (90 mg/kg) and xylazine (9 mg/lkg), an OVX was performed, and a osmotic pump was inserted under the skin. Briefly, a 28-day Alzet mini-osmotic dispensing 750 ng/day of 17β-estradiol benzoate was placed subcutaneously in each rat. Then to simulate the proestrus surge all the rats receiving a 17β-estradiol containing pump were injected subcutaneously with 2.5 μg of 17β-estradiol benzoate in 0.1 ml sesame oil every fifth day, as previously described (Kramer and Bellinger, 2009). Similar to intact female rats, the estrogen replacement regimen results in a baseline (i.e., diestrus) plasma 17β-estradiol concentrations of 7-10 pg/ml and a proestrus plasma estradiol concentration of 60-70 pg/ml (Butcher et al., 1974; Kramer and Bellinger, 2009). Administering this pattern of estrogen results in a vaginal smear pattern similar to that of intact rats (Kramer and Bellinger, 2009). Two advantages of this hormone model are one, we can determine estradiol's effect without the confounding effects of other sex hormones (e.g., progesterone, LH, FSH) and second, we know the physiological estradiol concentration (Proestrus [E2] or Diestrus [E2]) when performing nociceptive recordings or tissue sampling. In the present study we choose to assay nociception and molecular expression in the diestrus and proestrus groups as this is the greatest time separation from high to low estradiol levels in the estrous cycle and these time points were expected to provide the greatest differences.

Ligature placement (Experiment 1 and 2)

Bilateral ligature of the tendon attachment for the anterior superficial portion of the masseter muscle was completed after giving ketamine (90 mg/kg) and xylazine (9 mg/lkg) anesthesia. Ligature was completed by placing two 4.0 chromic gut ligatures, spaced 2-3.0 mm apart around the tendon (Guo et al., 2010). Surgical access to the tendon was from the interior of the mouth. The incision in the mouth was closed with a single 5.0 polyglycolic acid suture using a 13 mm 3/8 needle. Sham operated rats received the same surgery but the tendon was not ligated.

Meal Duration Assay (Experiment 1 and 2)

The rats were housed individually in sound-attenuated chambers equipped with photobeam computer-activated pellet feeders (Med Assoc. Inc., East Fairfield, VT). The feeders were loaded with 45 mg rodent chow pellets (Product No. FO 165, Bioserv, Frenchtown, NJ). When a rat removed a pellet from the feeder trough, a photobeam placed at the bottom of the trough was no longer blocked, signaling the computer to drop another pellet. The computer recorded the date and time and kept a running tally of the total daily food consumption. In these analyses, a meal was defined using a 10-minute end of meal criterion (i.e., a meal was bracketed before and after by a 10 minute period of no pellets being taken) and the minimum meal size was set at 135 mg [i.e., 3 pellets] (Castonguay et al., 1986). Meal patterns (i.e., food intake, meal size, meal number and meal duration) were calculated using Med Assoc. Inc. and proprietary software (Bellinger et al., 2007; Guan et al., 2005; Kerins et al., 2005; Kramer and Bellinger, 2009; Kramer et al., 2010). Meal duration has been shown to be a continuous, non-invasive measure of orofacial nociception (surface and deep) in undisturbed male and female rats (Kerins et al., 2005; Kerins et al., 2003). Rats take most of their meals during the dark phase and thus we report the dark phase meal duration.

Mechanical Sensitivity Testing (Experiment 1 and 2)

At five day increments the animals were removed from the feeding chambers and a series of calibrated von Frey filaments were applied to the skin above the ligatured tendons. A swipe of the paw or an active withdrawal of the head from the probing filament was defined as a response. Each von Frey filament was applied five times at intervals of a few seconds. The response frequencies (EF50) were calculated as described by Ren's group (Guo et al., 2010). Briefly, the response frequencies [(number of responses/number of stimuli) × 100%] to a range of von Frey filament forces were determined and a stimulus-response frequency curve was plotted. After a non-linear regression analysis, the half maximal response [i.e., EC50 values calculated by Prism 5.0 software (GraphPad, Inc., La Jolla, CA), here termed EF50] was calculated from the stimulus response curve. A smaller EF50 value indicates greater sensitivity and a larger value indicates less sensitivity. Testing was completed by an investigator blinded to experimental manipulation.

Guide cannula placement surgery (Experiment 2)

Ten days before OVX surgery (see Table 1) rats were anesthetized with a mixture of ketamine (75 mg/kg) and xylazine (0.5 mg/kg) (Bellinger and Tillberg, 1997) and guide cannulas (22 GA, Plastics One Inc, Roanoke, VA) were stereotaxically (David Kopf Instruments, Tujunga, California) placed in the TG bilaterally using coordinates 4.3 mm posterior of Bregma, 3.4 mm lateral of the midline at a depth of 9.5-10.3 mm from the dura (Paxinos and Watson, 2007). Stereotaxic coordinates were established in a preliminary trial by injecting India ink through the cannulas followed by a postmortem histological examination and in all animals cannula placement was confirmed by x-ray (Puri et al., 2012).

Trigeminal ganglion infusions (Experiment 2)

Twenty-four hours after ligature surgery the rats were anaesthetized with 5% isoflurane and infused bilaterally with 5 μl of control or siRNA solution over a 5 min period (see Table 1 for infusion day) using an infusion pump (KDS Model 310 Plus, Holliston, MA). Gabrα6 siRNA (2.5 μg) or control siRNA (2.5 μg) (Invitrogen, Carlsbad, CA) was mixed with linear polyethyleneimine (PEI) (In vivo JetPEI, Cat# 201-10, PolyPlus Transfection, Illkirch, France) to increase the transfection efficiency (Boussif, 1995). The ratio of cationic PEI amines (N) to nucleic acid phosphates (P) was N/P=6. This amount of siRNA and PEI was based on a previous study where siRNA knockdown was performed in vivo (Puri et al., 2012). The control siRNA had no homology to any known gene and was identified as Silencer Negative Control #1 siRNA (5'-AGUACUGCUUACGAUACGGtt-3', 5'-CCGUAUCGUAAGCAGUACUtt-3') and the Gabrα6 siRNA sequence was sense (5'-GGAACGAUCCUGUACACCAtt-3'), anti-sense (3'-UGGUGUACAGGAUCGUUCCat-5'). Animals were alert and mobile less than 5 minutes after removal from isoflurane. A 30-40% reduction in Gabrα6 expression within the TG has been verified in several studies using this exact method (Kramer and Bellinger, 2013; Puri et al., 2012).

Western blot analysis (Experiment 2)

On the day of sacrifice (see Table 1) the rats were killed with carbon dioxide gas and quickly decapitated. The TG from each rat just rostral of V1 and 2 mm caudal of V3 was dissected after removal of the brain. A slice of caudal brainstem was also collected from a tissue block that included a 2-mm segment beginning 4-5 mm caudal to the obex. This tissue block included the caudal laminated (Vc) and upper cervical spinal cord (C_1). The tissue block was turned coronally and the superficial portion of the Vc was harvested by taking punches with a 15-gauge non-beveled needle. The tissues were homogenized in 300 μl of T-Per tissue protein extraction reagent containing Halt Protease Inhibitor (Thermo Scientific, Rockford, IL). The homogenates were centrifuged for 10 min at 4°C and decanted. Total protein in the supernate was determined in each sample using a BCA protein assay (Thermo Scientific) and 15 μg of total protein was loaded into each well of a 4-12% Bis-Tris acrylamide gel (Invitrogen). The gel was electrophoresed at 200 volts for 35 minutes and the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was rinsed in Tris-buffered saline containing 0.1% Tween-20 and then blocked for one hour in this buffer containing 5% milk. Membranes were placed in the block solution with either an antibody against 1) Gabrα6 (1:2000) or 2) Gabrβ1 (1:2000) or 3) Gabrβ2 (1:1000) or 4) Gabrβ3 (1:2000) or 5) against β-actin (1:2000, rabbit polyclonal, Cell Signaling, Beverly, MA). The membranes were first probed with anti-Gabrα6, stripped (Re-blot Plus Mild, Millipore, Billerica, MA) and then probed with anti-β-actin. A second set of western blots was probed with anti–Gabrβ1, stripped; probed second with anti–Gabrβ2, stripped; probed third with anti–Gabrβ3, and finally probed with anti-β-actin. For the Gabrα6 antibody, a two hour incubation was used, whereas for each subsequent antibody the incubation was performed overnight at 4°C. The membranes were rinsed three times after the incubation with the primary antibody and then HRP conjugated goat anti-rabbit antibody (1:500, Cell Signaling) was added; followed by incubation at room temperature for 90 minutes. After incubation in this secondary antibody the membranes were rinsed three times and reacted

with the ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire UK). After exposure of the membrane to film, the bands were quantitated with Image J software (NIH, Bethesda, MD). The area and mean values were multiplied to obtain the optical density of the band. Values were reported as a ratio of the optical density of the protein band of interest (i.e., Gabrα6, Gabrβ1, Gabrβ2, Gabrβ3) divided by the optical density of the β-actin band. Controls in which the primary antibody was not included showed no signal (data not shown). Previous studies showed pre-absorption of the Gabrα6 antibody with a Gabrα6 peptide resulted in elimination of the Gabrα6 specific band (Puri et al., 2012).

The $GABA_A$ receptor is made up of two β subunits that can comprise three different isoforms β1-3 (Connolly et al., 1996; Schofield et al., 1989; Ymer et al., 1989). It has been reported that a decrease in Gabrα6 expression attenuates the number of $GABA_A$ receptors and that by quantitating the amount of the β subunits one can determine changes in the total amount of $GABA_A$ receptor (Nusser et al., 1999). In the present study, changes in total $GABA_A$ receptor levels were determined in the TG and the Vc-C1 by performing western blots for the Gabrβ1, β2 and β3 subunits for each treatment group.

Immunofluorescent staining of TG tissue from proestrus rats

Three rats were treated with a proestrus dose of estradiol as in Experiment 1 and had their TG removed the day of estradiol injection. TG tissues were then stored in a 25% sucrose solution, frozen, and cryosectioned. The 30 mm cryosections were placed on Histobond slides (VWR international, Irving TX), blocked with a PBS solution containing 5% normal goat serum and 0.3% Triton-X 100 for 2 hours at room temperature. The slides were then incubated in a primary antibody solution overnight at 4°C. Primary antibodies consisted of a rabbit ERα antibody (Santa Cruz Biotechnology, Dallas TX; 1:50) and a goat Gabrα6 antibody (Santa Cruz Biotechnology, 1:500) . Primary antibodies were diluted with PBS, 1% BSA and 0.3% Triton X-100. After incubation in primary antibody the slides were rinsed four times in PBS and 0.3% Triton X-100 for a total of 60 min and placed for 2 hours in a 1:500 dilution of secondary antibodies in PBS and 0.3% Triton X-100. Secondary antibodies included rabbit anti-goat 488 and goat anti-rabbit 568 (Invitrogen). After rinsing the slides four times in PBS for a total of 60 min, the slides were then mounted with Fluoromount-G mounting medium (Electron Microscopy Sciences, Hatfield, PA) containing Hoechst dye. The fluorescent signal was captured using a Nikon fluorescent microscope and NIS-Elements imaging software and a Photometrics CoolSnap K4 CCD camera (Roper Scientific, Inc, Duluth, GA).

Statistics

Filament data was analyzed using a Mann-Whitney test. Meal pattern and gene expression effects were determined pre and post-ligature by ANOVA with the independent variables of surgery (sham/ligature), drug (control siRNA/Gabrα6 siRNA) and hormone status (Diestrus [E2], Proestrus [E2]) with the dependent variables being either meal patterns or the optical density of the specific band divided by the β-actin band. Groups with significant main effects were further analyzed by a Bonferroni post-hoc test (ABstat software, V1.94 or

Graph Pad Prism version 5.0, Graph Pad, La Jolla, CA). Data with $p < 0.05$ was considered significant. All values were presented as mean ± SEM.

Results

Experiment 1

Nociceptive response measurements—Five days after ligature of the masseter tendon meal duration was significantly lengthened $F(1,50)=125$, $P<0.001$ (Fig. 1A) but treatment with a high concentration of estradiol (Proestrus [E2]) significantly reduced the nociceptive response as seen by a shortened meal duration $F(1, 50)=5.34$, $P<0.05$ (Fig. 1A). A significant interaction was observed between ligature and 17β-estradiol treatment, $F(1, 50)=4.9$, $p<0.01$. Mechanical sensitivity testing showed the ligature group had an increased nociceptive response. In accord with the meal duration measurements, the elevated (Proestrus [E2]) concentration of estradiol attenuated the nociceptive filament response (Fig. 1B).

Experiment 2

Nociceptive response measurements—As found in Experiment 1 tendon ligature significantly lengthened the meal duration post-day 1 and post-day 5 [Fig. 2A, $F(1, 43)=56.6$, $p<0.001$] and treatment with a high concentration of estradiol (Proestrus [E2]) significantly attenuated the nociceptive response as measured by a shortened meal duration post-day 1, $F(1, 24)=4.7$, $P<0.05$ (Fig. 2A). Three days after infusion of Gabrα6 siRNA into the TG (post-day 5) meal duration increased in the high estradiol treatment group (Proestrus [E2]) infused with Gabrα6 siRNA [$F(1, 43)=6.27$, $p<0.05$] but not control siRNA (Fig. 2A), which showed the rats had a heightened nociceptive response when Gabrα6 was reduced. No significant interaction between ligature and siRNA treatment was observed $F(1,43)=0.4$, $p=0.53$. Moreover, infusion with Gabrα6 siRNA had no significant effect on food intake, meal size and meal number.

The filament force used to generate a response after ligature significantly decreased (Fig. 2B), which demonstrated the rats were experiencing more nociception. On post-day 5, the increased concentration of estradiol significantly attenuated this nociceptive response (compare the Proestrus [E2]/ligature/control siRNA group to the Diestrus [E2]/ligature/control siRNA group, Fig. 2B). Consistent with the meal duration results, infusion with Gabrα6 siRNA resulted in a greater nociceptive response in comparison to rats infused with control siRNA (compare the Proestrus [E2]/ligature/control siRNA group to the Proestrus/ligature/Gabrα6 siRNA group, Fig. 2B.

Quantitation of Gaba receptor subunits—Gabrα6 expression significantly increased in the TG [$F(1, 31)=9.3$, $p<0.01$] (Fig. 3A) and Vc-C_1 [$F(1, 23)=6.9$, $p<0.05$] (Fig. 3B) after treatment with a high concentration of 17β-estradiol. Infusion of Gabrα6 siRNA into the TG significantly reduced Gabrα6 expression in both the TG [$F(1, 31)=31.5$, $p<0.001$] (Fig. 3A) and Vc-C_1 [$F(1, 23)=12.5$, $p<0.01$] (Fig. 3B). Ligature did not affect expression. No significant interactions were observed.

Total $GABA_A$ receptor content in the TG and Vc-C1 was estimated by western blot measurement of Gabrβ1, β2 and β3 for each treatment group, see Materials and Methods (Nusser et al., 1999). The main effect for infusion of Gabrα6 siRNA into the TG was a reduction in Gabrβ1 expression in both the TG [$F(1, 22)=11.3$, $p<0.01$, $n=4$] and Vc-C_1 [$F(1, 17)=4.4$, $p<0.05$, $n=3$] suggesting that total $GABA_A$ receptor content was reduced. Conversely, after administering a high estradiol concentration Gabrβ1 expression significantly increased in the TG [$F(1, 22)=6.0$, $p<0.01$] but not the Vc-C_1 [$F(1, 17)=0.003$, $p=0.95$]. Ligature had no effect on Gabrβ1 expression. Also, no significant interactions were observed with exception of ligature and estradiol treatment in the TG, $F(1, 22)=7.4$, $p<0.05$. No significant changes in β2 and β3 expression were observed in the TG and Vc-C_1 due to either siRNA treatment, ligature or estradiol administration (data not shown). Post-hoc testing showed no significant effects for Gabrβ1, β2 and β3.

Expression of ERα and Gabrα6 in TG cells of proestrus rats—We have previously shown that ERα and Gabrα6 are expressed in neurons of the TG (Puri et al., 2011) but double labeling has not been performed. In Figure 4 cells with a neuronal morphology stained for both ERα and Gabrα6 in the TG.

Discussion

Treating with a high concentration of estradiol decreased the nociceptive response and increased Gabrα6 expression in female and male rats (Kramer and Bellinger, 2009; Puri et al., 2011). Infusing Gabrα6 siRNA into the trigeminal ganglia reduced Gabrα6 expression in the trigeminal ganglia and trigeminal nucleus caudalis in female and male rats (Puri et al., 2012). For the first time this study shows that reducing Gabrα6 expression partially reversed the decreased nociceptive response normally observed after treating with a high concentration of estradiol. This reversal could be due to decreased levels of $GABA_A$ receptor present in the TG and caudalis regions as indicated by a lower amount of Gabrβ1 after Gabrα6 siRNA infusion.

Guo, et al., 2010 showed that masseter tendon ligation can induce hypersensitivity as measured with a filament assay. In the present study, mechanical sensitivity testing showed a significant response after masseter tendon ligation. Meal duration measurements were confirmed by the mechanical sensitivity testing assay, in that, a high concentration of 17β-estradiol treatment significantly attenuated hypersensitivity in the ligated rats. While both the meal duration assay and filament assay measure orofacial nociception and hypersensitivity, respectively (Guo et al., 2010; Bellinger et al., 2007) there are some differences. Mechanical sensitivity testing produces a mechanical pressure induced response, whereas meal duration is a functional measure of nociception. Nevertheless, both assays showed that high levels of 17β-estradiol significantly attenuate orofacial nociception/hypersensitivity when compared to rats with low levels of 17β-estradiol (diestrus group).

Meal duration and von Frey measurements indicated that the rats were more sensitive following Gabrα6 siRNA infusion after estradiol treatment, reflecting involvement of Gabrα6 in orofacial nociception/hypersensitivity. In this study we observed a reduction in both Gabrα6 and Gabrβ1 in the TG and Vc-C1 after Gabrα6 siRNA infusion. A reduction in

Gabrα6 expression can reduce $GABA_A$ receptor content (Jones et al., 1997; Nusser et al., 1999), furthermore a reduction in a specific $GABA_A$ receptor subunit can lead to a reduction in the amount of functional $GABA_A$ receptor, inhibiting the activity of GABA (Glykys et al., 2008; Nusser et al., 1999; Wei et al., 2003; Yu et al., 1996). For example, a recent study demonstrated that loss of a single subunit can affect $GABA_A$ receptor function (Witschi et al., 2011). Since orofacial nociceptive responses are inhibited by Gabaergic signaling (Cairns et al., 1999; Viggiano et al., 2004) a decrease in $GABA_A$ receptor content would be expected to result in a greater nociceptive response (Almond et al., 1996; Viggiano et al., 2004) and this was the observed in Gabrα6 siRNA treated female rats given a high dose of estradiol.

Gabrα6 expression was greater in sham and ligatured female rats after administering a high (proestrus) dose of estrogen, consistent with previous results in rats with inflammatory TMJ arthritis (Puri et al., 2011; Puri et al., 2012). Moreover, in the group treated with a high dose of estradiol (Proestrus [E2]) there was an increase in Gabrβ1 in the TG which could reflect an increase in $GABA_A$ content. A mechanism to explain this hormone effect would be that estradiol increased the amount of functional $GABA_A$ receptor that then sensitized neurons to the inhibitory effects of GABA. This inhibitory mechanism could include action of estradiol in TG neurons containing both ERα and Gabrα6. These inhibitory effects are known for the trigeminal pathway, for example GABA in the Vc-C_1 region has the potential to bind $GABA_A$ receptors and result in neuronal inhibition at both the superficial and deep laminae (Anderson et al., 2009), moreover treatment with a $GABA_A$ agonist in the Vc region can block excitatory responses in the trigeminal nucleus interpolaris and Vc region (Bereiter et al., 2002).

We propose that estradiol alters the nociception/hypersensitivity by altering Gabrα6 expression centrally. In a gene array study of the TMJ, trigeminal ganglia and caudalis, gene expression was compared between OVX rats given a low (diestrus) dose of estradiol or high (proestrus) dose of estradiol. No change in Gabrα6 was observed in the TMJ tissue, which included the condyle, disc, and retrodiscal tissue (Puri et al., 2011). In contrast, this study showed the amount of Gabrα6 did change in the TG and to a smaller extent in the Vc-C1 region. Higher Gabrα6 gene expression in the TG and Vc-C1 after treatment with a high dose of estradiol occurred in parallel to a reduction in the nociceptive/hypersensitivity response but it is unknown if the higher estrogen- dependent Vc-C1 expression is the result of primary TG nerve terminals (projecting to the caudalis) having a higher amount of Gabrα6 or if cells in both in the TG and Vc-C1 express a greater amount of Gabrα6 in response to estradiol cell autonomously. In either case, estradiol leads to a reduced nociceptive/hypersensitivity response and adecrease in Gabrα6 expression in the Vc-C1 but not in the TMJ consistent with a central mechanism. This idea is supported by recent studies that show estradiol alters gabergic signaling in the Vc-C1 to effect the TMJ response (Tashiro et al., 2014).

Estradiol's effect on orofacial pain is clearly complex but epidemiological studies have indicated that women seek treatment for TMD more often than men (LeResche, 1997) and a decrease in estrogen during the late luteal phase and low estrogen during menstruation correlate to heightened pain responses in TMD patients (LeResche et al., 2003).

Polymorphisms in the estrogen receptor increase the risk of women developing TMD, such that, women have a significantly higher risk of moderate or severe pain when polymorphisms are present in this receptor (Kang et al., 2007; Ribeiro-Dasilva et al., 2009). Estrogens have also been shown to decrease sensitivity to noxious masseter muscle stimuli (Smith et al., 2006) suggesting estradiol attenuates the pain response under certain conditions, consistent with the decreased ligature response observed in the proestrus rats.

We interpret the reduction in the meal duration response from post-day 1 to post- day 5 as being due, in part, to Gabrα6 expression. If the meal duration response after ligature was due exclusively to Gabrα6 one would have expected the meal duration in the proestrus/ligature/Gabrα6 siRNA group to be the same on post-day 1 and post-day 5, assuming Gabrα6 siRNA knockdown was 100%. Instead the meal duration in the proestrus/ligature/Gabrα6 siRNA group trended lower on post-day 5 as compared to post-day 1, consistent with the idea that other mechanisms could contribute to the meal duration response after ligature.

In summary, Gabrα6 expression increased when rats received a proestrus dose of 17β-estradiol. Rats given a proestrus dose of 17β-estradiol also showed a decreased nociceptive response. Gabrα6 siRNA infusion, which has been shown to reduce Gabrα6 expression, increased the nociceptive response and reversed the attenuating effect of estradiol. This is the first time that estrogen has been shown to affect the nociceptive response in a myogenic model of TMJ pain and the results suggest that the decreased nociceptive response after estradiol administration is due, in part, to the increased inhibitory action of $GABA_A$ receptors containing the Gabrα6 subunit.

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List of Abbreviations

Gabrα6	$GABA_A$ receptor subunit alpha 6
Gabrβ1	$GABA_A$ receptor subunit beta 1
Gabrβ2	$GABA_A$ receptor subunit beta 2
Gabrβ3	$GABA_A$ receptor subunit beta 3
OVX	ovariectomized
PVDF	polyvinylidene fluoride
SEM	standard error of the mean
siRNA	small interfering RNA
TASM	tendon attachment of the anterior superficial portion of the masseter muscle
TMD	temporomandibular joint disorder

TMJ	temporomandibular joint
TG	trigeminal ganglia
Vc	trigeminal nucleus caudalis
C_1	upper cervical region

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Highlights

-Proestrus levels of estradiol decreased the nociceptive response and increased Gabrα6 expression

-Trigeminal ganglia (TG) infusion of Gabrα6 siRNA reduced its expression in the TG and nucleus caudalis

-Reducing Gabrα6 reversed the decreased nociceptive response in rats injected with estradiol

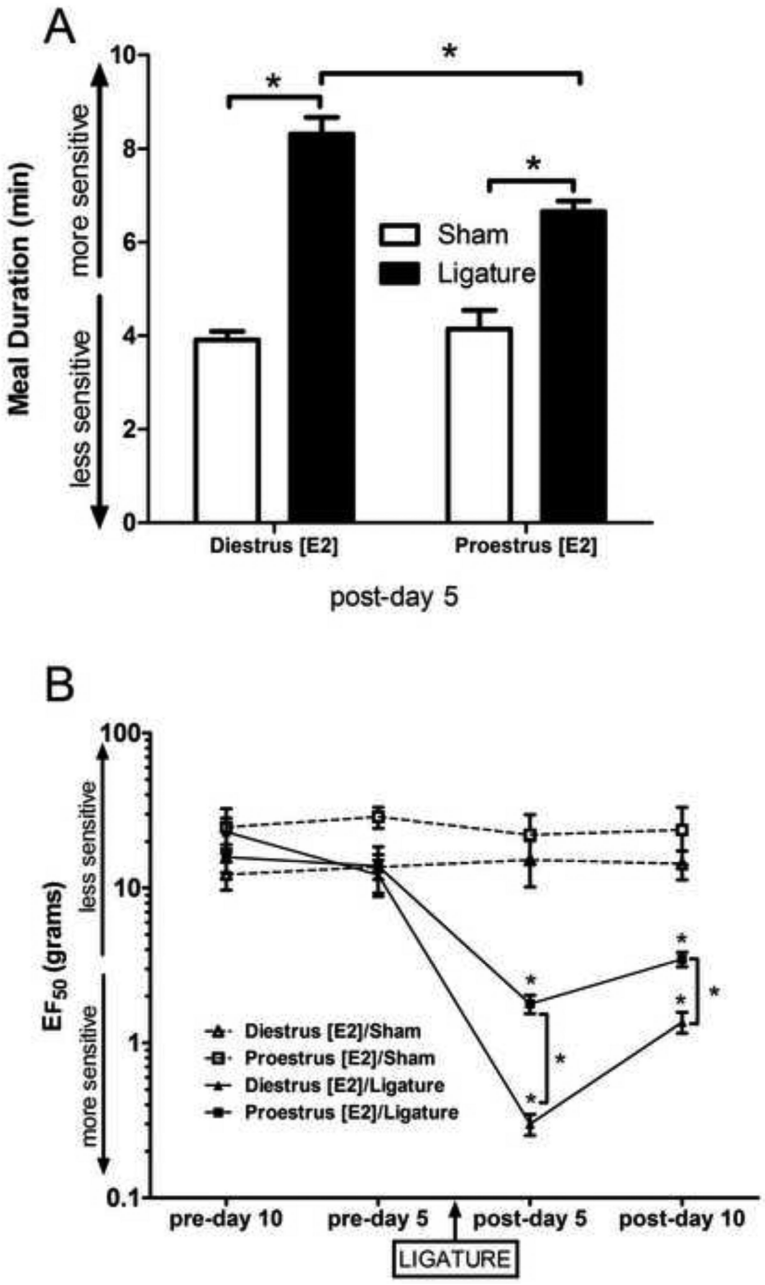

Figure 1. Administration of 17β-estradiol attenuates the nociceptive response following ligation of the masseter tendon

Ovariectomized rats were given a high and low dose of estradiol and the TASM was ligatured. The nociceptive response was measured; panel A shows meal duration values and panel B shows von Frey filament testing over the masseter. The term Diestrus [E2] indicated 17-β estradiol was administered to provide a plasma concentration equivalent to the diestrus phase and the term Proestrus [E2] indicated 17-β estradiol was administered to an equivalent dose found in the proestrus phase. Ligation of the masseter tendon or a sham surgery was performed at the time indicated in Panel B by “LIGATURE”. Measurements were made 5 (pre-day 5) or 10 days (pre-day 10) before ligature or 5 (post-day 5) or 10 days (post-day 10)

after ligature. Values are the mean ± SEM, differences are indicated by *= P<0.05. In panel B significant differences (p<0.05) between the respective sham and ligature groups are indicated by asterisk over the data point. There were 10 animals per group for the sham diestrus and proestrus groups and 15 animals for the ligature diestrus and proestrus groups.

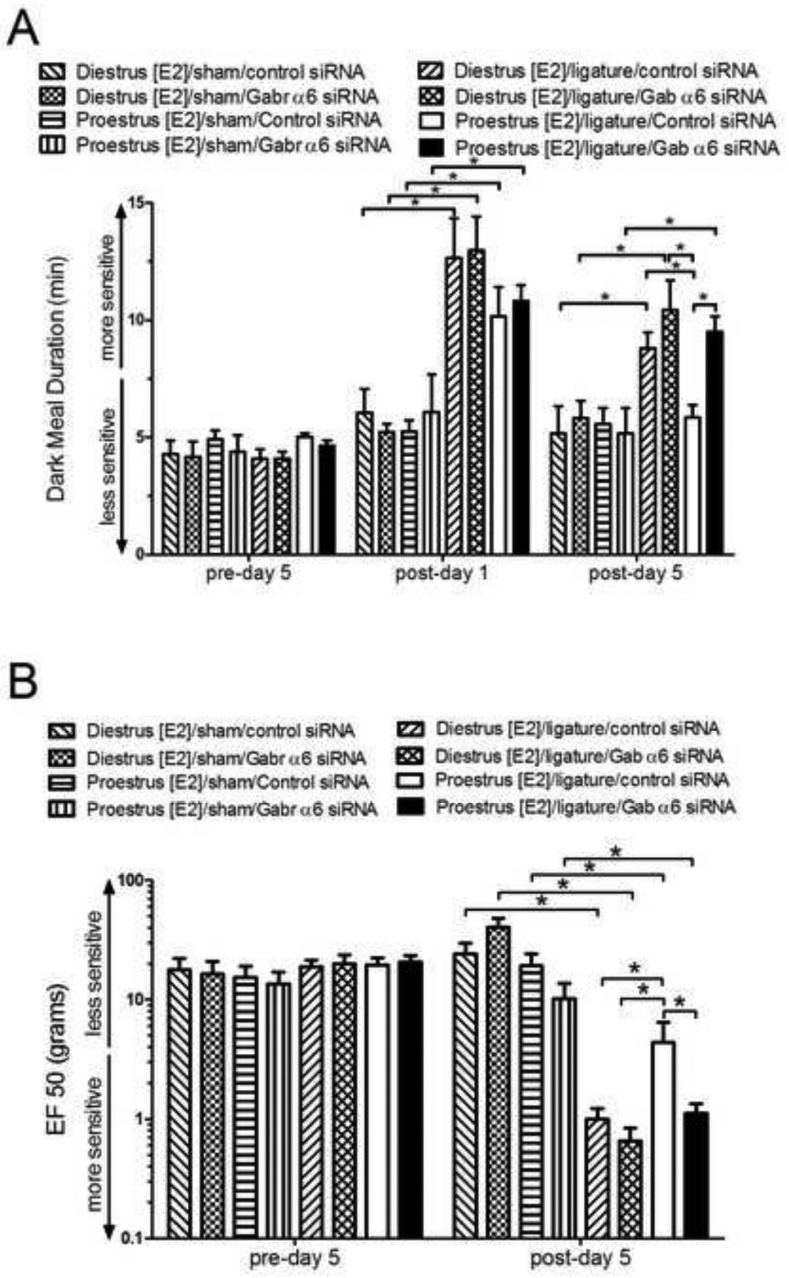

Figure 2. Gabrα6 siRNA infusion increased the nociceptive response in estradiol treated rats

In this experiment siRNA was infused into the TG of rats given a high and low dose of estradiol. Meal duration (Panel A) and von Frey filament measurements (Panel B) were recorded for rats 5 days before TASM ligature (pre-day 5), 1 day after ligature (post-day 1) and/or 5 days after ligature, which was 3 days after siRNA infusion (post-day 5). See Table 1 for a timeline. Values are the mean ± SEM, *=$p<0.05$. There were 7 animals per group.

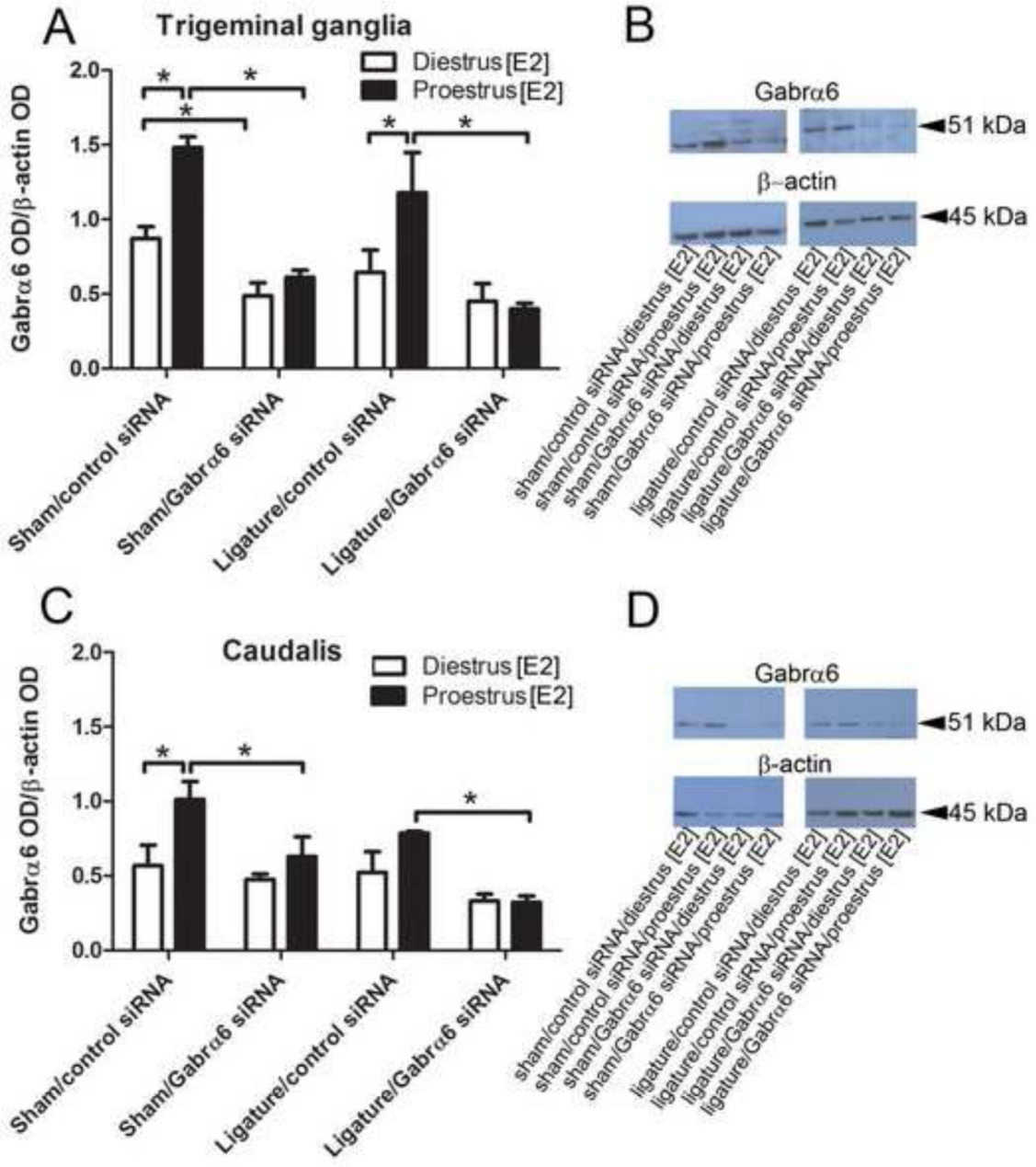

Figure 3. Gabrα6 siRNA infusion reduced Gabrα6 expression in the TG and the Vc-C_1 in estradiol treated rats 5 days after a ligation or sham operation of the masseter tendon

Gabrα6 siRNA infusion into the TG reduced Gabrα6 expression on western blots reported as a ratio of the optical density of the Gabrα6 band divided by the optical density of the β-actin band. Panel A shows the values for the TG and Panel C show shows the values for the Vc-C_1. Representative western blot images for the TG and Vc-C_1 are shown in panels B and D, respectively. Values are the mean ± SEM, differences are indicated by *= $p<0.05$. There were 5 animals per group for the trigeminal ganglia western blots and 4 animals per group for the Vc-C1 western blots.

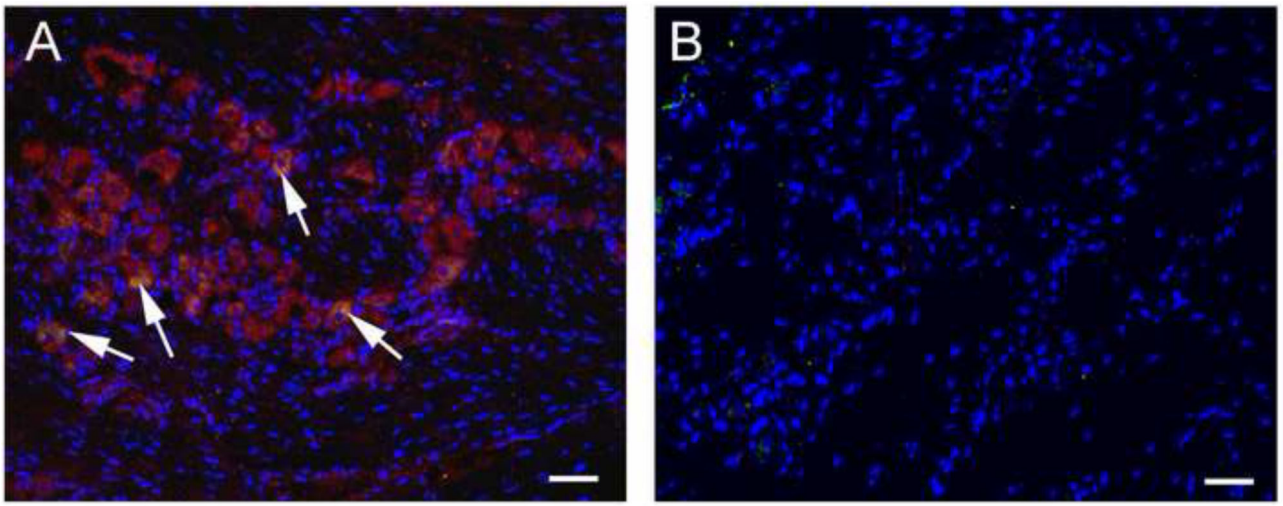

Figure 4. Detection of ERα and Gabrα6 expression in TG cells of proestrus rats

A) Fluorescent staining for ERα (red) and Gabrα6 (green) indicated that a few cells contain both ERα and Gabrα6 (yellow, arrows); representative image of three proestrus rats. Cell nuclei are stained blue with Hoechst dye. B) No fluorescent signal was observed without addition of primary antibodies. Bar = 20 μM.

Table 1

Treatment Group Timeline (note: 5 day estrus cycle)

Ligature Day	TREATMENT
−33	■ Female Sprague-Dawley rats arrive from Harlan Industries ■ Rats for Experiment #2 allowed to acclimatize to new surroundings (14 hour light phase:10 hour dark phase)
−29	■ Guide cannula surgery was performed on rats in Experiment #2
−24	■ Female Sprague-Dawley rats arrive from Harlan Industries ■ Rats for Experiment #1 allowed to acclimatize to new surroundings (14 hour light phase:10 hour dark phase)
−20	■ Place rats in computerized feeding chambers
−19	■ Filament test to acclimate animals to testing modality ■ Females are ovariectomized and an endogenous concentration of hormone was administered by implanting Alzet pumps dispensing 750 ng/day of 17β-estradiol benzoate (cycled rats)
−15	■ Half the cycled rats were injected with 2.5 μg of S17β-estradiol benzoate in 0.1 ml sesame oil [Proestrus Group]
−14	■ Half the cycled rats were injected with 2.5 μg of 17β-estradiol benzoate in 0.1 ml sesame oil [Diestrus Group]
−10	■ Half the cycled rats were injected with 2.5 μg of 17β-estradiol benzoate in 0.1 ml sesame oil [Proestrus Group] ■ Filament test on all the rats in all treatment groups for Experiment #1 and Experiment #2 (pre-day 10)
−9	■ Half the cycled rats were injected with 2.5 μg of 17β-estradiol benzoate in 0.1 ml sesame oil [Diestrus Group]
−5	■ Half the cycled rats were injected with 2.5 μg of 17β-estradiol benzoate in 0.1 ml sesame oil [Proestrus Group] ■ Filament test on all the rats in all treatment groups for Experiment #1 and Experiment #2 (pre-day 5).
−4	■ Half the cycled rats were injected with 2.5 μg of 17β-estradiol benzoate in 0.1 ml sesame oil [Diestrus Group]
1	■ A ligature is placed around the tendon of the masseter muscle for half of the rats in each group, the other half of the rats receive a sham operation ■ Half the cycled rats were injected with 2.5 μg of 17β-estradiol benzoate in 0.1 ml sesame oil [Proestrus Group]
2	■ Half the cycled rats were injected with 2.5 μg of 17β-estradiol benzoate in 0.1 ml sesame oil [Diestrus Group] ■ Infused siRNA in all rats in the Diestrus and Proestrus groups in Experiment #2
5	■ Half the cycled rats were injected with 2.5 μg of 17β-estradiol benzoate in 0.1 ml sesame oil [Proestrus Group] ■ Filament test on all the rats in all treatment groups for Experiment #1 and Experiment #2 (post-day 5)

Ligature Day	TREATMENT
6	■ Half the cycled rats were injected with 2.5 μg of 17β-estradiol benzoate in 0.1 ml sesame oil [Diestrus Group] ■ Sacrifice rats and collect TG and Vc-C_1 tissue for rats in Experiment #2
10	■ Half the cycled rats were injected with 2.5 μg of 17β-estradiol benzoate in 0.1 ml sesame oil [Proestrus Group] Filament test on all the rats in all treatment groups for Experiment #1 (post-day 10)
11	■ Sacrifice rats from Experiment #1