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Sex-specific modulation of spinal nociception by α_2 -adrenoceptors: differential regulation by estrogen and testosterone

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Abstract

Sex-related differences in antinociception produced by the activation of α_2 -adrenoceptors (α_2 -ARs) have been reported, however, the precise role of gonadal steroids is still unknown. Hence, we hypothesized that estrogen and testosterone modulate antinociceptive effects of clonidine (an α_2 -AR agonist) on N-methyl-D-aspartate (NMDA)-and heat-induced spinal nociception. We also investigated whether estrogen-or testosterone alter the expression of α_{2A} -adrenoceptors in the spinal cord. Sprague-Dawley (SD) rats were implanted with PE10 cannulae in the intrathecal space of the lumbosacral spinal cord and divided into male, proestrous and diestrous female, ovariectomized (OVX), estradiol-treated OVX (OVX+E), castrated male (GDX), testosterone (GDX+T) and estrogen-treated GDX (GDX+E) groups. Clonidine dose-dependently inhibited NMDA-induced scratching behavior in the male and OVX groups but to a significantly lesser extent in the OVX+E group. It also increased the tail withdrawal latency in the male, OVX, diestrous and GDX+T groups but not in the OVX+E, proestrous, GDX and GDX+E groups. Levels of α_{2A} -AR mRNA were significantly higher in the OVX, estradiol-treated OVX, GDX and GDX+E animals. In contrast, α_{2A} -AR protein levels were higher in estradiol-treated OVX, GDX, GDX+T and GDX+E animals as compared to the male. Indeed, no correlations were observed between changes in the mRNA or protein levels of α_{2A} -AR and behavioral observations. These results support our hypothesis that sex-related differences in α_2 -AR-mediated modulation of spinal nociception are gonadal hormone-dependent: estrogen attenuates antinociceptive effects in females whereas testosterone is required for the expression of antinociception in males. In addition, results also revealed that the mechanism of action of gonadal hormones may not involve a global alternation in expression of α_{2A} -AR in the spinal cord. Estrogen-induced attenuation of α_2 -AR-mediated inhibition of nociception could contribute to the higher prevalence of pain syndromes in women.

Keywords

sex differences; pain; tail flick; clonidine; analgesia; spinal cord

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Sex-related differences in the perception of pain and the response to analgesics have been reported with females having lower thresholds for pain (Notermans, 1966; Neri et al., 1984; Berkley, 1997; Riley et al., 1999; Rollman et al., 2000; reviewed in Keogh et al., 2002) and a larger number of pain sites as compared to males (Fillingim and Maixner, 1995; Berkley, 1997; Fillingim et al., 1999). In addition, many pain syndromes/disorders such as fibromyalgia, rheumatoid arthritis, irritable bowel syndrome, temporomandibular joint disorder, and migraine headaches are more prevalent in women and are affected by alterations in sex hormones (Fillingim and Maixner, 1995; Marcus, 1995; Unruh, 1996; Berkley, 1997; LeResche et al., 1997; reviewed in Riley et al., 1999). Fluctuations in sex hormones during different phases of the menstrual cycle also affect pain perception and modulation in females (Tedford, 1977; Kuczmierczyk, 1986; Hapidou, 1988; Fillingim et al., 1995; reviewed in Riley et al., 1999). α_2 -adrenoceptors (α_2 -ARs) are G-protein-coupled receptors (GPCRs) that, like other GPCRs eg. opioid and GABA receptors, have been shown to elicit antinociception in humans and animals (reviewed in Yaksh, 1985; Kayser et al., 1992; Guilbaud, 1992; Eisenach, 1995; Millan, 1997, 2002). α_2 -AR-agonists administered systemically, intrathecally or epidurally, produce antinociception through spinal and supraspinal mechanisms (reviewed in Eisenach et al., 1996; Asano et al., 2000; Mitrovic et al., 2003) and have been utilized in treating several severe pain states, such as cancer pain and neuropathic pain (Tamsen and Grodh, 1984; Glynn et al., 1986; Glynn and O'Sullivan, 1995; Eisenach et al., 1996; Dobrydnjov et al., 2005). Gene manipulation studies have shown that among three subtypes of α_2 -ARs: α_{2A} , α_{2B} and α_{2C} , the α_{2A} subtype predominantly mediates antinociceptive effects in rodents (Lakhani et al., 1997; Stone et al., 1997; Wang et al., 2002). Sex-specific modulation of nociception by opioid receptors has been previously demonstrated by us (Zhang et al., 1998; Flores et al., 2001; Claiborne et al., 2006) and others (Cicero et al., 1996; Kepler et al., 1991; Bartok and Craft, 1997; Kest et al., 2000; reviewed in Miaskowski et al., 2000; Mogil et al., 2000; Zubieta et al., 2002; Wang et al., 2002; Stoffel et al., 2003). However, there is limited evidence regarding sex-related differences in α_2 -AR-induced spinal antinociception with one study showing diminished and enhanced antinociceptive effect of clonidine (an α_2 -AR agonist; administered intraperitoneally) after castration and ovariectomy respectively, but no change during various estrous cycle stages (Bodnar et al., 1991), and the other (Liu and Gintzler, 1999) showing involvement of α_2 -ARs in pregnancy-induced antinociception. Further, the precise role of gonadal hormones on sex-specific modulation of spinal nociception by α_2 -adrenoceptors still remains unknown. Hence, we hypothesized that the activation of α_2 -ARs in the spinal cord produces sex-specific, gonadal steroid (estrogen and testosterone)-dependent modulation of nociception. We also investigated whether changes in the sensitivity to α_2 -AR agonist could be explained by changes in spinal expression of message or protein encoding the α_{2A} subtype of α_2 -ARs.

EXPERIMENTAL PROCEDURES

Animals

Adult Sprague-Dawley male, female, ovariectomized female (OVX) and castrated male (GDX) rats (225–275 g) were obtained from Harlan Sprague Dawley Inc. (Harlan, Indianapolis, IN). They were kept in the animal care facility at Meharry Medical College certified by the American Association for the Accreditation of Laboratory Animal Care (AALAC) under a 12-hour light/dark cycle (lights on: 7 AM; lights off: 7 PM) and had free access to food and water. All experimental protocols were approved by Institutional Animal Care and Use Committee of Meharry Medical College and conformed to the guidelines established by the National Research Council Guide for the Care and Use of Laboratory Animals and the International Association for the Study of Pain (IASP). All efforts were made to minimize stress to the animals and the number of animals used.

Intrathecal Implantation of Cannulae

A PE-10 cannula (Intramedic, Clay Adams, Parsippany, NJ; dead volume 10 μ l) was implanted intrathecally in the lumbosacral region of the spinal cord (modified from Yaksh and Rudy, 1976) in animals anesthetized with ketamine and xylazine (72 and 4 mg/kg respectively, i.p.) as detailed previously (Claiborne et al., 2006). Briefly, the animal's head was secured in a stereotaxic frame (David Kopf) and an incision was made in the head/neck region to expose the atlanto-occipital membrane. A small opening was made in the dura with a 18-gauge needle through which the cannula was inserted in the subarachnoid space and gently pushed 8.5 cm caudally to reach the lumbosacral enlargement and was secured with dental cement. The incision was sutured, animals were kept on a heating blanket (36°C) until they recovered from anesthesia and were then returned to their home cages. Nociceptive testing began after a 5–7 days recovery period. A standard vaginal smear test was used to determine the estrous cycle stage. Females were cannulated in the diestrous phase and experiments were performed only after they underwent two regular cycles. Our laboratory has previously demonstrated that estrous cycle stages determined by vaginal smear correlate very well with the serum estradiol levels measured using radioimmunoassay (Claiborne et al., 2006). OVX and GDX rats were cannulated two weeks after the removal of gonads.

Estrogen/Testosterone replacement

Hormone injections were performed at least two weeks after the removal of gonads. OVX rats received a single subcutaneous injection of estradiol benzoate (1 ng, 1, 10 or 100 μ g/100 μ l sesame oil) 48 hours prior to nociceptive testing. This method of administration has been used in neuroendocrinology (Berglund et al., 1988; Priest et al., 1995) as well as the pain fields (Ji et al., 2003, 2005). The 100 μ g dose of estradiol was used in majority of experiments because it is known to reliably induce lordosis behavior in rats (Clark, 1993a). Our laboratory has previously reported a time course of dose-dependent changes in serum estradiol levels in OVX rats (Nag and Mokha, 2006) as well as in normally cycling females (Claiborne et al., 2006). Levels of estradiol were found to be within the physiological range 48 hours after estradiol injection. GDX males were given a single subcutaneous injection of testosterone propionate (250 μ g/100 μ l sesame oil) 24 hours prior to nociceptive testing. This dose of testosterone has been shown to produce maximum copulatory behavior in castrated male rats (Clark, 1993b).

N-methyl-D-aspartic acid (NMDA)-induced nociceptive testing

NMDA (4 nmol/10 μ l) (Aanonsen and Wilcox, 1987) was administered intrathecally followed by 10 μ l of sterile saline. NMDA microinjection induced nociceptive scratching, biting and licking behaviors that were restricted to the hind limb region and lasted for up to 4 minutes. The number of scratches/bites, latency and duration were manually recorded. Slightly higher doses of NMDA produced frankly noxious behavior including vocalization and attempts to escape in our pilot studies and were excluded.

Drugs

Various doses (0.875, 1.75, 2.5, 3.5, and 7 μ g/5 μ l) of clonidine, an α_2 -AR agonist, were intrathecally administered in separate groups, 15 minutes prior to NMDA microinjection or after baseline readings in the tail flick experiment. Doses greater than 7 μ g produced apparent sedative effects in our pilot studies and were excluded. Yohimbine (30 μ g/15 μ l; Solomon et al., 1989; Nag and Mokha, 2004; Nag and Mokha, 2006), a selective α_2 -AR antagonist, was injected intrathecally 5 minutes prior to the administration of clonidine. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in sterile saline.

Tail-flick test

The tail-flick test (D'Amour and Smith, 1941) was conducted using a tail-flick analgesiameter (Model 33T, IITC Life Science, Woodland Hills, CA). Animals were loosely restrained in a Plexiglas cylinder and habituated for 1 hr. prior to testing. The heat stimulus was applied to the dorsal surface of the tail through a radiant heat source at two separate spots located between 5–9 cm from the tip of the tail to prevent sensitization produced by heating a single spot repeatedly. A change in the tail temperature, whether induced by alteration in hormones (Hosono et al., 2001; Opas et al., 2006) or by fluctuation in room temperature and other factors, could be a confounding factor in the tail flick test (Hole and Tjolsen, 1993). To avoid this, a trigger temperature setting was used to automatically pre-warm the tail to 32°C in all groups before the intense heating started. The heat intensity was set to produce an average baseline tail-flick latency of 3.5–6.0 seconds. A cutoff latency of 15 seconds was used to prevent tissue damage. Tail-flick latencies were automatically recorded at 5 min intervals before and after drug injections.

Tissue collection, RT-PCR and Taqman real-time qPCR

Animals were deeply anesthetized with sodium pentobarbital (100 mg/kg; i.p.), the lumbar spinal cord was collected in RNA later (0.5 ml; Ambion, Austin, TX) and stored at –80°C until further processing. RNA was isolated using the Rneasy lipid tissue mini kit (QIAGEN, Inc., Valencia, CA) and purified with Dnase I. cDNA was synthesized using the Reverse-transcriptase cDNA archive kit (Applied Biosystems, Foster City, CA). Expression of the α_{2A} -AR subtype was measured by real-time PCR (Icycler, Biorad Laboratories, Hercules, CA) using a Taqman gene expression kit from Applied Biosystems. A two-step PCR protocol was used: 50°C, 2 min; 95°C, 10 min; 95°C for 15 seconds and 50°C for 1 min for 40 cycles. Manufacturers' instructions were followed through each step. GAPDH gene expression was measured similarly and was used to normalize α_{2A} -AR expression. Relative expression was calculated using cycle threshold (Ct) values by Pfaffl method (2001).

Western blotting

Lumbar spinal cord tissue was collected as described above and total tissue lysate was prepared in CLB buffer (0.5 ml/100 mg tissue; 10 mM Tris, 300 mM sucrose, 1 mM AEBSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM EDTA, 1 $\mu\text{g}/\mu\text{l}$ aprotinin, 1 $\mu\text{g}/\mu\text{l}$ iodoacetamide, 1 $\mu\text{g}/\mu\text{l}$ leupeptin, 1 $\mu\text{g}/\mu\text{l}$ σ -phenanthroline) by homogenization for 1 min using a dounce homogenizer. A crude plasma membrane fraction was prepared by spinning the total homogenate at 7500 RPM (5 min, 4°C) discarding the pellet, spinning again at 13000 RPM (1 hr., 4°C) and resuspending the pellet in 100 μl cold PBS as described by Galan et al. (2004). Total protein content of each sample was determined using a Lowry based DC protein assay kit (Biorad). The absorbance was read at 750 nm on a Beckman DU-360 spectrophotometer and protein concentrations were calculated against a BSA standard curve. For western blotting, equal amount of protein (15 $\mu\text{g}/15 \mu\text{l}$ Lammeli sample buffer; Biorad) for each sample was subjected to SDS-PAGE (Biorad; 10% separating gel; 50V for 1 hr, 80V for 2 hr) after heating at 65°C in a shaking water bath. Protein bands were transferred onto nitrocellulose membrane, blocked for 1 hr and probed with β -actin (1:500; Sigma-Aldrich, St. Louis, MO) or α_{2A} -AR (1:300; C10; Daunt et al., 1997; obtained from Dr. Brian Kobilka, Stanford University) rabbit polyclonal primary antibodies for 1 hr at room temperature. After washing, the blots were incubated with HRP-conjugated donkey anti-rabbit secondary antibody (1:3000 for α_{2A} -AR and 1:5000 for β -actin; GE Healthcare, Pittsburgh, PA), developed using chemiluminescence (Santa Cruz Biotechnology, Santa Cruz, CA) and exposed to x-ray films. X-ray films were scanned and densitometry was performed on the bands using Labworks Image Acquisition and Analysis Software (Bioimaging Systems, Upland, CA). Immunoblotting experiments for α_{2A} -AR were performed in the laboratories of Drs. Timothy Angelotti and Brian Kobilka

after determining that the commercially available antibody for α_{2A} -AR, used initially, did not bind specifically to the α_{2A} -AR (based on immunoblotting in tissues from α_{2A} -KO mice ; data not shown).

Data Analysis

All data were analyzed using SPSS (SPSS, Inc., Chicago, IL). Behavior data were submitted to analysis of variance (ANOVA) with appropriate between-(Group, Drug) and within-(Time) group factors, and dependent variables (number of scratches, latency, duration, tail withdrawal latency). Data from tail flick time course experiments was analyzed by repeated measures ANOVA followed by Fisher's LSD post hoc test. Additionally, area under the curve (AUC) was calculated by trapezoid method using Prism (Graphpad Software, Inc., San Diego, CA) for time course plots to obtain a single measure of the overall response. The data obtained from western blotting and real-time PCR studies were analyzed by one-way ANOVA. A post-hoc test (Fisher's LSD) was employed for intergroup comparisons where necessary and only when ANOVA yielded a significant main effect. A p-value of <0.05 was considered significant. Data were plotted as mean \pm SEM.

Results

Estrogen-dependent sex-specific inhibition of NMDA-induced nociception by α_2 -ARs

Intrathecal microinjection of NMDA (4 nmol/10 μ l) produced comparable nociceptive scratching behavior in the male, OVX and OVX+E groups. There were no significant differences in both measures of NMDA-induced nociception, i.e. the number of scratches (Fig. 1A) and the duration of scratching (Fig 1B) between these groups.

To determine whether activation of α_2 -ARs in the spinal cord produces sex-specific modulation of nociception, clonidine (7 μ g/5 μ l), an α_2 -AR agonist, was administered intrathecally 15 minutes prior to the administration of NMDA. Significant main effects of Group [$F_{2,44}=4.83$ (scratches) $p<0.05$] and Drug [$F_{2,44}=47.60$ (scratches); 15.45 (duration); $p<0.05$] as well as an interaction between the two factors ($F_{4,44}=3.14$; $p<0.05$; scratches only) were obtained on ANOVA. Post-hoc comparisons revealed that clonidine significantly reduced the number of scratches (Fig. 1A) and duration (Fig. 1B) in all three groups in comparison to their respective controls. However this effect of clonidine was significantly less pronounced in the OVX+E group in comparison to that in the male and OVX groups ($p<0.05$). The ability of yohimbine (30 μ g/15 μ l; i.t.; 5 minutes before clonidine), a selective α_2 -AR antagonist, to block the antinociceptive effects of clonidine in all groups (Fig. 1A, B) is consistent with the interpretation that the antinociception produced by clonidine is due to the activation of spinal α_2 -ARs. As shown in Fig. 1C, the effect of clonidine was dose-dependent in all groups. ANOVA yielded significant main effects of Group ($F_{2,90}=6.24$; $p<0.05$) and Drug ($F_{5,90}=18.00$; $p<0.05$) on NMDA-induced scratches. Post-hoc comparisons revealed that clonidine significantly reduced the number of scratches in the male and OVX at three higher doses (2.5 – 7.0 μ g). However, only the highest dose of clonidine (7 μ g) significantly reduced the number of scratches in the OVX+E group and this reduction was significantly less than that in clonidine-treated male or OVX groups ($p<0.05$). These findings indicate that observed differences in the ability of clonidine to suppress NMDA-induced nociceptive behavior are sex-related and not due to changes in the dose-response to clonidine.

Estrogen-dependent sex-specific modulation of heat-evoked nociception by α_2 -ARs

To assess whether sex-related differences in the antinociceptive properties of clonidine are specific to NMDA-induced nociception, a heat-evoked tail flick test was employed. Normally cycling females were also included in this study in order to assess whether

estrogen-induced reduction in the antinociceptive effects of clonidine can be observed in normally cycling females in parallel with fluctuations in estrogen levels. Groups representing phases of higher (proestrous) and lower (diestrous) endogenous estrogen levels were included. Significant main effects of Group ($F_{4,65}=37.67$; $p<0.001$) and Drug ($F_{2,65}=202.83$; $p<0.001$) as well as an interaction between these two factors ($F_{6,65}=25.91$; $p<0.001$) were obtained on ANOVA. Post hoc comparisons revealed that while baseline tail withdrawal latencies were comparable in all groups, clonidine (7 $\mu\text{g}/5\mu\text{l}$) significantly increased tail withdrawal latencies in the male, OVX and diestrous groups (with lower estrogen levels). However, clonidine had no effect in the OVX+E and the proestrous groups (with higher estrogen levels). Yohimbine fully antagonized antinociceptive effects of clonidine in the male, OVX and diestrous groups (Fig. 2A).

Estrogen attenuates antinociceptive effects of clonidine in a dose-dependent manner

To determine the “threshold” level of estradiol that diminishes the antinociceptive effects of clonidine, the effects of clonidine were tested on heat-evoked nociception in OVX animals treated with estradiol benzoate over a range of 1 ng to 100 μg . As a comparator, levels of estrogen detected in proestrous rats correlate with those measured 48 hr. after the administration of a single 100 μg dose of estradiol. ANOVA yielded significant main effects of Group ($F_{3,40}=24.44$; $p<0.001$) and Drug ($F_{1,40}=53.41$; $p<0.001$) as well as an interaction between these two factors ($F_{3,40}=22.41$; $p<0.001$). Post hoc comparisons revealed that clonidine, as expected, produced a significant increase in tail-flick latency in the OVX group (Fig. 2B). Estradiol injected at a 1 ng dose did not alter this effect, whereas treatment with 1, 10 or 100 μg estradiol fully blocked the effect of clonidine ($p<0.05$).

Time course of the sex-specific modulation of heat-evoked nociception

To determine whether the sex-specific antinociceptive effect of clonidine on thermal nociception is present throughout the duration of the antinociceptive response, a time course study was performed. The control baseline tail withdrawal latencies were comparable in the male, OVX, OVX+E, proestrous and diestrous groups (Fig 3A). Intrathecal administration of clonidine (7 $\mu\text{g}/5\mu\text{l}$) produced a significant increase in tail withdrawal latencies in the OVX and male groups, but not in the OVX+E group (consistent with data provided in Fig. 2A). Repeated-measures ANOVA yielded significant main effects of Time ($F_{11,165}=8.10$; $p<0.001$) and Group ($F_{4,15}=11.26$; $p<0.001$). Post hoc comparisons revealed that clonidine significantly increased the latencies starting at 5 min in the male and 10 min in the OVX group compared to their respective vehicle controls (all $p<0.05$). The antinociceptive effect of clonidine was maintained for the period of testing (90 min). In contrast, there was no effect of clonidine in the OVX+E group (Fig. 3A). Clonidine also significantly increased the tail withdrawal latencies in normally cycling females at the diestrous but not at the proestrous stage of estrous cycle (Fig. 3B). ANOVA yielded significant main effects of Time ($F_{11,99}=10.34$; $p<0.001$) and Group ($F_{2,9}=68.22$; $p<0.001$). The cumulative effect of clonidine over the entire time course, presented as area under the curve (Fig. 3D) was also significantly higher in the male, OVX and diestrous groups in comparison to the OVX+E, proestrous and the respective vehicle control groups ($F_{10,43}=17.11$, $p<0.001$). These findings are consistent with the interpretation that high levels of estrogen present naturally can also inhibit antinociceptive effects of clonidine.

Testosterone is required for the expression of the antinociceptive effect of clonidine in the male

To determine if testosterone plays a role in mediating antinociceptive effects of clonidine in the male, GDX males were administered testosterone (250 $\mu\text{g}/100\mu\text{l}$ sesame oil, s.c.) 24 hours prior to, or estradiol (100 $\mu\text{g}/100\mu\text{l}$ sesame oil) 48 hours prior to nociceptive testing. The antinociceptive effect of clonidine was abolished in GDX males (Fig. 3C). However,

clonidine produced a significant increase in the tail withdrawal latency in GDX males treated with testosterone propionate (GDX+T). In contrast, estrogen did not alter the lack of effect of clonidine in the GDX group. ANOVA revealed significant main effects of Time ($F_{11, 99} = 5.60$; $p < 0.001$) and Group ($F_{2, 9} = 12.85$; $p < 0.001$). Post-hoc comparisons revealed that clonidine significantly increased tail withdrawal latencies in the GDX+T group at all time points ($p < 0.05$), but not in the GDX and GDX+E groups. The baseline tail withdrawal latencies were comparable in all groups. As shown in Fig. 3D, AUC was significantly higher in the GDX+T group in comparison to the GDX and GDX+E groups ($F_{10, 43} = 17.11$, $p < 0.001$). These findings indicate that testosterone is required for the expression of the antinociceptive effects of clonidine in the male, whereas, as shown in Fig. 1–3, estrogen suppresses the effects of clonidine in the female.

Expression of the α_{2A} -AR subtype mRNA or protein in the spinal cord does not correlate with sex-specific modulation of pain

As mentioned earlier, there are three α_2 -AR subtypes (Bylund et al., 1992; Bylund et al., 1994; reviewed in Ruffolo, 1994; Hein and Kobilka, 1998; Saunders and Limbird, 1999), and it is the α_{2A} subtype, which principally mediates the antinociceptive effects of clonidine in the spinal cord (Lakhlani et al., 1997; Stone et al., 1997; Wang et al., 2002). To determine if changes in expression of the α_{2A} -AR could explain the effect of estrogen to suppress the antinociceptive effects of clonidine, the expression of the α_{2A} -AR gene relative to that of GAPDH was determined in the lumbosacral region of the spinal cord in the male, OVX, OVX+E (1 ng -100 μ g), proestrous, diestrous, GDX, GDX+T and GDX+E rats using real-time PCR. The male group was used as the control against which all other groups were compared. ANOVA revealed a significant main effect of Group ($F_{7, 32} = 14.65$; $p < 0.001$). Post hoc comparisons showed that α_{2A} -AR mRNA levels were significantly higher in OVX, 10 μ g estradiol-treated OVX, GDX and GDX+E groups in comparison to males (Fig. 4A). However, there was no significant difference between the male and OVX groups treated with 1 ng, 1 μ g and 100 μ g estradiol, proestrous, diestrous and GDX+T groups. At 1 μ g estrogen, no changes in receptor mRNA expression occurred, even though, at this dose, behavioral effects, in terms of tail withdrawal latencies, are maximal (Fig. 2B and 4A). These data emphasize that there is no correlation between estrogen- or testosterone-induced changes in the level of α_{2A} -AR mRNA (Fig. 4A) and the behavioral suppression of α_{2A} -AR induced antinociception (Fig. 1, Fig 2 and Fig 3).

Protein level of α_{2A} -AR, relative to that of β -actin, was determined in the lumbosacral region of the spinal cord by western blotting using a polyclonal antibody against the α_{2A} -AR subtype developed in the laboratory of Dr. Brian Kobilka (Fig. 4B). ANOVA revealed a significant main effect of Group ($F_{10, 32} = 6.64$, $p < 0.05$). Post hoc comparisons showed that α_{2A} -AR protein levels were elevated in the OVX+E (10 μ g), GDX, GDX+T and GDX+E groups. However, there were no significant differences in the OVX, OVX+E (1 ng, 1 μ g and 100 μ g), proestrous and diestrous groups in comparison to the male. These changes do not correlate with either the mRNA expression in the spinal cord (Fig. 4A) or the observed antinociceptive effects in behavioral studies (Fig 1–3).

Discussion

This is the first comprehensive study to demonstrate that activation of α_2 -ARs in the spinal cord produces gonadal hormone-dependent, sex-specific antinociception. Estrogen attenuates antinociception in the female whereas testosterone is required for the expression of antinociception in the male. Attenuation of α_2 -AR-mediated antinociception by estrogen was observed in response to exogenous estrogen in ovariectomized animals as well as in normally cycling females in parallel with fluctuations in endogenous estrogen levels.

Further, it was the presence of testosterone, and not the low levels or the absence of estrogen in the male, that is necessary for the expression of antinociception.

In the present study, activation of α_2 -AR in the spinal cord produced sex-specific modulation of the NMDA-induced nociceptive scratching behavior. The NMDA-induced scratching behavior is related to nociception and not itch for several reasons: 1) the sensations of itch and pain have been shown to be distinct and antagonistic. Itch is mediated by itch selective C primary afferent fibers and a subset of itch selective spinothalamic neurons that do not respond to noxious mechanical or thermal stimuli (Schmelz et al., 1997 and Andrew and Craig, 2001); 2) slightly higher concentrations of NMDA produced frankly noxious behavior which was evident from vocalization responses and attempts to escape; 3) similar concentrations of NMDA that produce the scratching behavior also produce a hyperalgesic response to thermal stimuli, which is blocked by mu opioid agonists (Aanonsen and Wilcox, 1987; Wilcox, 1988).

The present study also revealed that α_2 -AR-mediated sex-related differences occur not only in response to NMDA-induced nociceptive behavior but also in response to thermal nociception. Clonidine produced a significant antinociceptive effect in the male, OVX, and diestrous animals, but not in the proestrous or the OVX+E animals. While exogenous estrogen dose-dependently attenuated antinociceptive effects of clonidine in OVX group, high levels of endogenous estrogen attenuated this effect in proestrous females compared to diestrous females (lower estrogen levels). Such bidirectional effects of estrogen in modulating activity of opioid receptors have been reported in animals (Claiborne et al., 2006) and human studies (Smith et al., 2006). Sex-related differences in antinociception produced by systemic injection of clonidine (Bodnar et al., 1991, Mitrovic et al., 2003) have been previously reported. Our results advance these previous findings in important ways. First, we tested the effects of clonidine applied locally (i.t.) in the spinal cord. Previous studies used systemic clonidine injection that may affect multiple spinal as well as supraspinal mechanisms. Blockade of endogenous α_2 -AR antinociception by intrathecal yohimbine in hormone-induced or normal pregnancy has been shown (Liu and Gintzler, 1999) but that may be affected by an altered hormonal milieu (sustained high levels of estrogen and progesterone) in addition to other changes present during a complex condition like pregnancy. Second, we demonstrated that it is estrogen that decreases the antinociceptive effect of α_2 -AR activation in the female. Third, we demonstrated that testosterone is required for the expression of antinociception in the male. Bodnar et al. (1991) concluded their study with the results derived from castration and did not substantiate their findings by hormonal replacement. In addition, they briefly mentioned (without providing any data) that estrous cycle did not alter clonidine's effect. On the other hand Mitrovic et al. (2003) reported sex differences in G protein-coupled, inwardly rectifying K^+ channel 2 (GIRK2) knock out and wild type mice without investigating the role of sex steroids. Fourth, we revealed for the first time that the modulation of α_2 -AR antinociception is not paralleled by changes in levels of mRNA or protein of the α_{2A} -AR subtype in the spinal cord of the rat.

The action of estrogen in the present study i.e. attenuation of α_2 -AR-mediated analgesia, was similar to those from our previous study with another GPCR – ORL1 (Claiborne et al., 2006). However, estrogen's actions may not be generalizable and may depend upon GPCR type, route of drug administration and therefore the site of action (spinal versus supraspinal), genotype and source of the experimental animals. Indeed, supraspinal morphine (a mu-opioid receptor agonist) injection produced most potent antinociception in the proestrous phase (high estrogen) relative to the met-diestrus phases (low estrogen) in SD rats from Charles River Laboratories (Kepler et al., 1989; Shane et al., 2007), whereas Bernal et al.

(2007) reported the most potent morphine antinociception in the diestrous phase relative to the estrous phase in SD rats from Taconic Farms.

Estrogen receptors (ER α and ER β) are known to be present in the dorsal horn of the spinal cord (Amanduson et al., 1995; Shughrue et al., 1997; Amanduson et al., 1999; 1998; VanderHorst, et al., 2005). Therefore, estrogen can directly attenuate the α_2 -AR-mediated antinociception by a number of possible mechanisms including, a) decreased expression of the α_{2A} gene, and b) decreased coupling of α_{2A} receptors to G proteins (Gi/Go). We investigated whether estrogen down regulates the levels of α_2 -AR mRNA or protein. Results, however, indicate that there was no clear correlation between estrogen-induced molecular changes and behavioral findings. Therefore, one has to look for mechanisms beyond the estrogen-induced down-regulation of the α_{2A} -AR gene/protein. Although estrogen has been shown to decrease the α_{2A} -AR mRNA levels and α_{2A} -AR binding density and elevate G protein coupled receptor kinase (GRK2) in the cerebral cortex (Karkanias et al., 1997), it has also been shown to not alter the α_{2A} -AR gene expression or protein levels in the hypothalamus. It rather stabilizes α_{2A} -AR phosphorylation, uncoupling the receptor from G proteins (Ansonoff and Etgen, 2001). It is, therefore, possible that estrogen may uncouple α_{2A} receptor from G proteins by promoting phosphorylation of the receptor in the spinal cord. Nevertheless, changes in mRNA and protein levels of α_{2A} -adrenoceptors observed in the present study are relative due to technical limitation and need to be further explored by additional studies with detailed measures e.g. time course, separation of spinal tissue (dorsal versus ventral and even within the dorsal horn) etc. to study these changes in diverse populations of neurons. The use of entire lumbar spinal cord tissue in the present study could mask estrogen-induced changes in gene expression that are neuron-specific, e.g. estrogen might differentially regulate gene expression depending on the neuron type i.e. down-regulates the α_{2A} -AR expression in projection neurons and/or up-regulates the expression in GABA containing interneurons. Testosterone induced up-regulation of the α_{2A} gene in the dorsal horn could contribute to the antinociception in the male since testosterone has been shown to elevate the levels of α_{2A} -AR mRNA in the cerebral cortex (Dygallo et al., 2002).

In conclusion, the results of this study supported our hypothesis that the activation of α_2 -ARs in the spinal cord produces sex-specific, gonadal steroid-dependent modulation of nociception. Our finding that estrogen suppresses α_{2A} -AR-induced antinociception in the spinal cord, which may contribute to the higher prevalence of pain syndromes in women, will aid in the design and administration of analgesic agents in women during the reproductive years and after menopause. Furthermore, our finding that testosterone is required for α_2 -AR-mediated antinociception in the male also suggest a strategy for enhancing antinociception in aging men.

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ABBREVIATIONS

α2-AR	α2-adrenoceptors
ANOVA	Analysis of variance
AUC	Area under the curve
Ct	Cycle threshold
DiE	Diestrous

GDX	Castrated male
GDX+E	Estradiol-treated castrated male
GDX+T	Testosterone-treated castrated male
GIRK2	G protein-coupled, inwardly rectifying K ⁺ channel 2
GPCR	G-protein coupled receptor
I.P.	Intraperitoneal
I.T.	Intrathecal
KF	Kölliker-Fuse
NMDA	<i>N</i> -methyl-D-aspartic acid
OVX	Ovariectomized
OVX+E	Estradiol-treated ovariectomized
ProE	Proestrous
RIA	Radioimmunoassay
S.C.	Subcutaneous

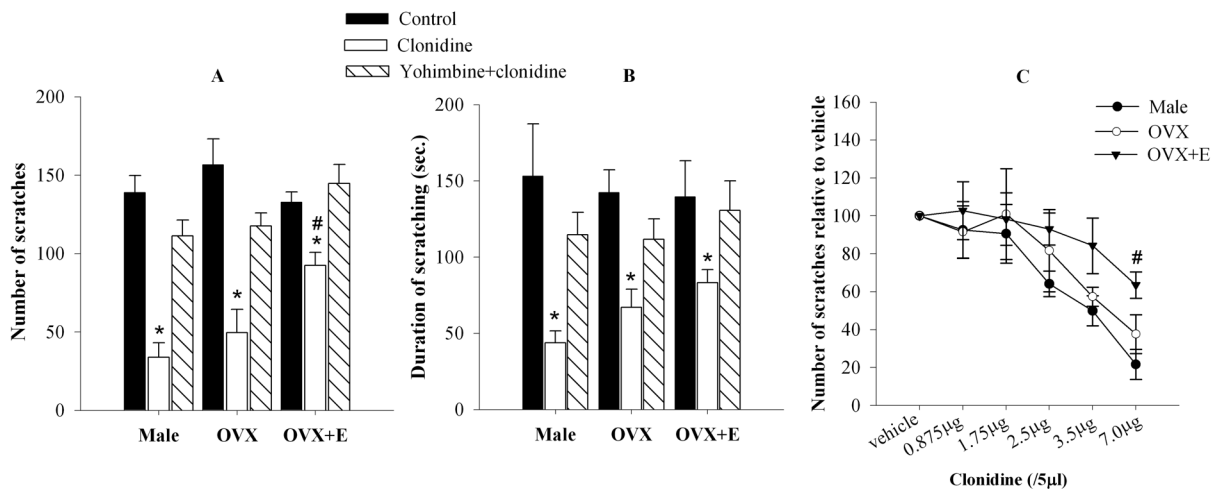


Figure 1. Activation of α_2 -ARs in the spinal cord produces sex-specific modulation of NMDA-induced nociception

A, B. Intrathecal administration of NMDA (4 nmol/10 μ l) resulted in scratching behavior (A, number of scratches; B, duration of scratching) in the hindlimb region, which was comparable in all groups (males, OVX and OVX+E). Clonidine (7 μ g/ 5 μ l), administered intrathecally 15 minutes prior to NMDA, significantly reduced the number of scratches and duration in all three groups. However this effect of clonidine was significantly less pronounced in the OVX+E group in comparison to that in the male and OVX groups. **C.** Clonidine produced dose-dependent antinociception in the male, OVX and OVX+E females. Three higher doses (2.5 – 7.0 μ g) of clonidine significantly reduced the number of scratches in the male and OVX groups. However, only the highest dose of clonidine (7 μ g) could significantly reduce the number of scratches in the OVX+E group and this reduction was significantly less than that in clonidine-treated male or OVX groups. * $p < 0.05$ compared to control; # $p < 0.05$ compared to clonidine-treated male or OVX groups.

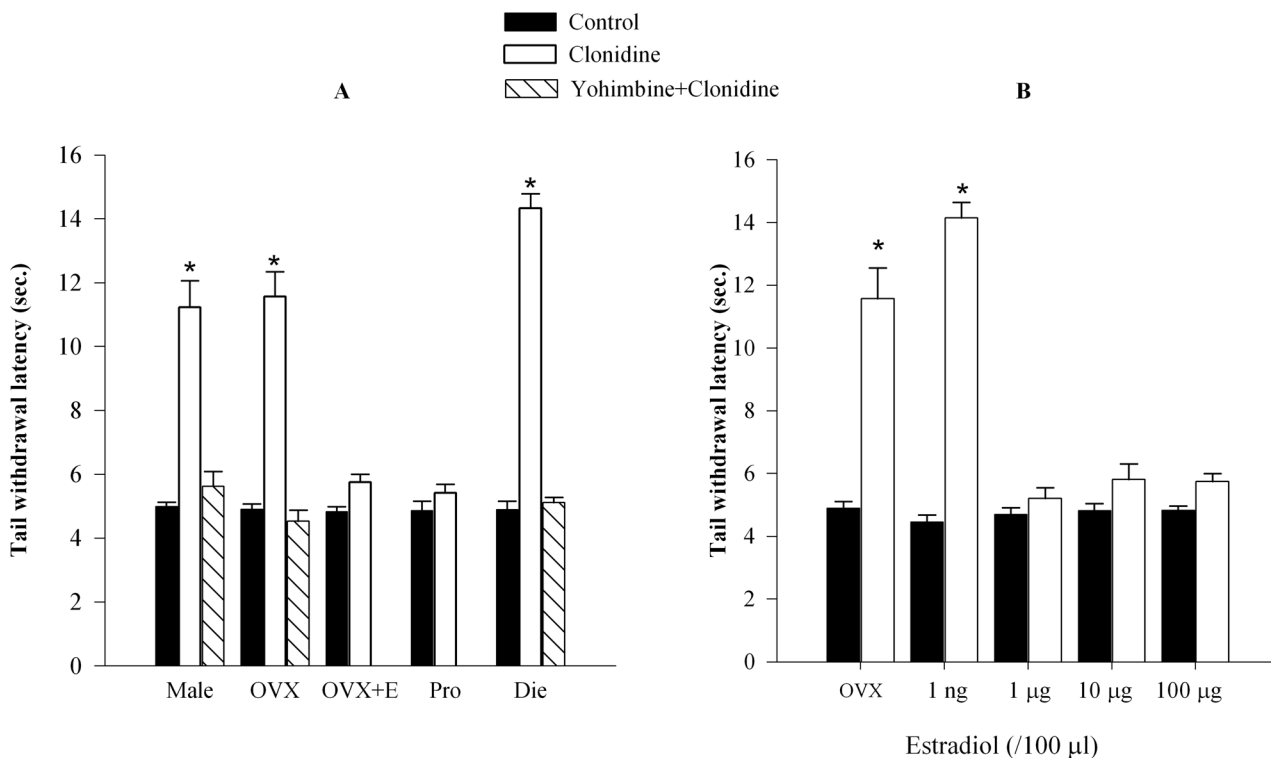


Figure 2. Clonidine increases tail withdrawal latency in male, OVX, diestrous, and in the presence of low, but not high levels of estradiol

A. Baseline tail withdrawal latencies were comparable in all groups. Clonidine (7 µg/ 5µl), administered 15 minutes prior to testing, significantly increased the tail withdrawal latencies in the male, OVX and diestrous groups (with lower estrogen levels), but not in the OVX+E and proestrous groups (with higher estrogen levels). Clonidine's effects were blocked by yohimbine in the male, OVX and diestrous groups. **B.** Estrogen dose dependently attenuated the antinociceptive effect of clonidine. It completely reversed the increase in tail withdrawal latencies produced by clonidine only at higher doses (1 µg, 10 µg or 100 µg/100 µl), but not at the lowest dose (1 ng). * $p < 0.05$ compared to control.

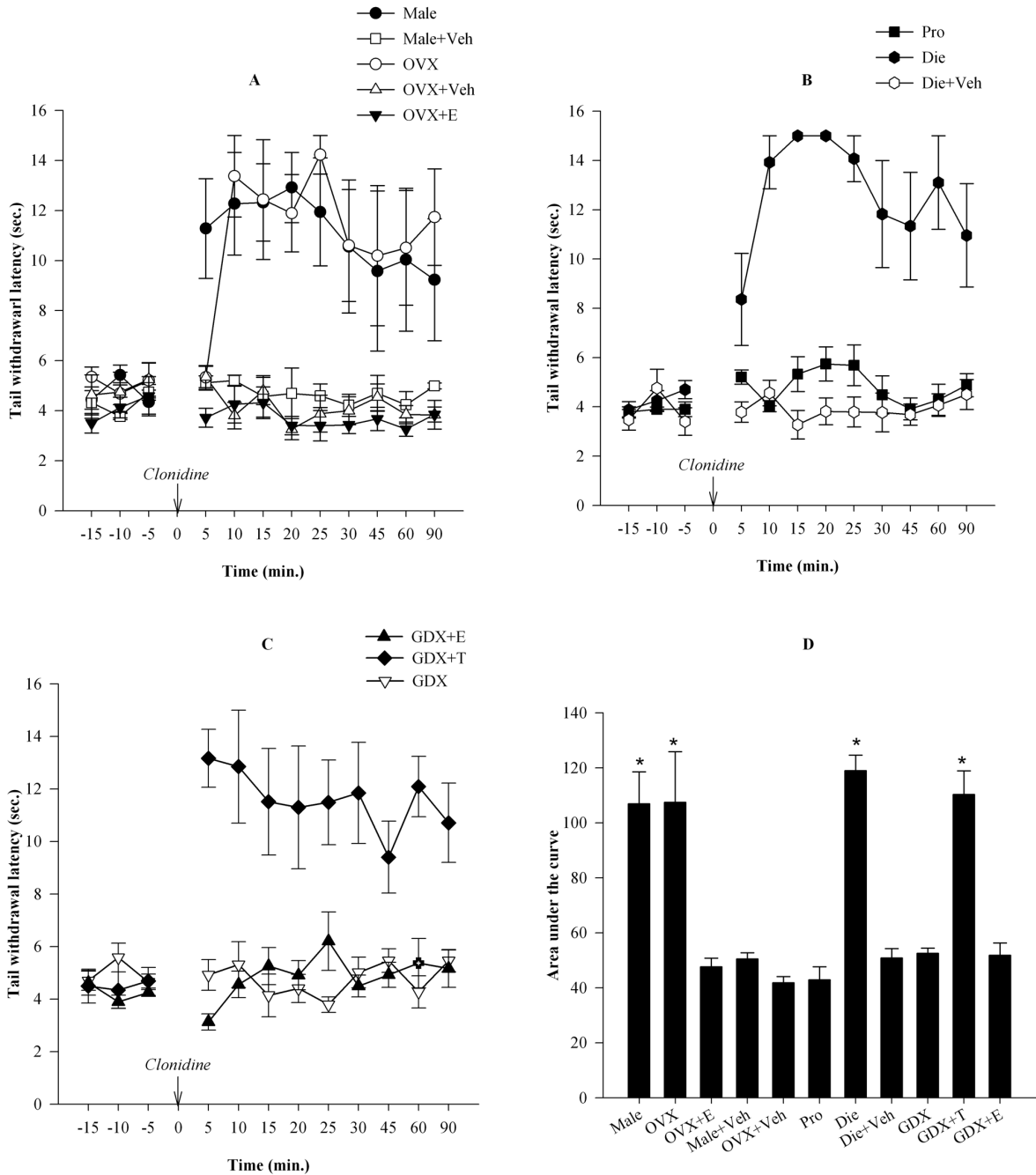


Figure 3. Estrogen attenuates whereas testosterone is required for the expression of antinociception produced by clonidine

A. Intrathecal administration of clonidine (7 μg / 5 μl) produced a significant increase in tail withdrawal latencies in OVX and male groups, but not in OVX+E groups. Clonidine significantly increased the latencies in the male and OVX groups, and maintained its effects for the period of testing (90 min). However, there was no effect of clonidine in the OVX+E group. **B.** In normally cycling females, clonidine significantly increased the tail withdrawal latencies at the diestrous stage, but not at the proestrous stage. **C.** Clonidine was completely ineffective in castrated males. Estradiol treatment did not alter this absence of effect, however, testosterone replacement in GDX males restored the antinociceptive effects of

clonidine. **D.** The cumulative effect of clonidine over time was determined using area under the curve analysis. AUC was significantly higher in the male, OVX, diestrous and GDX+T groups in comparison to the OVX+E, proestrous, GDX, GDX+E and vehicle control groups. * $p < 0.05$

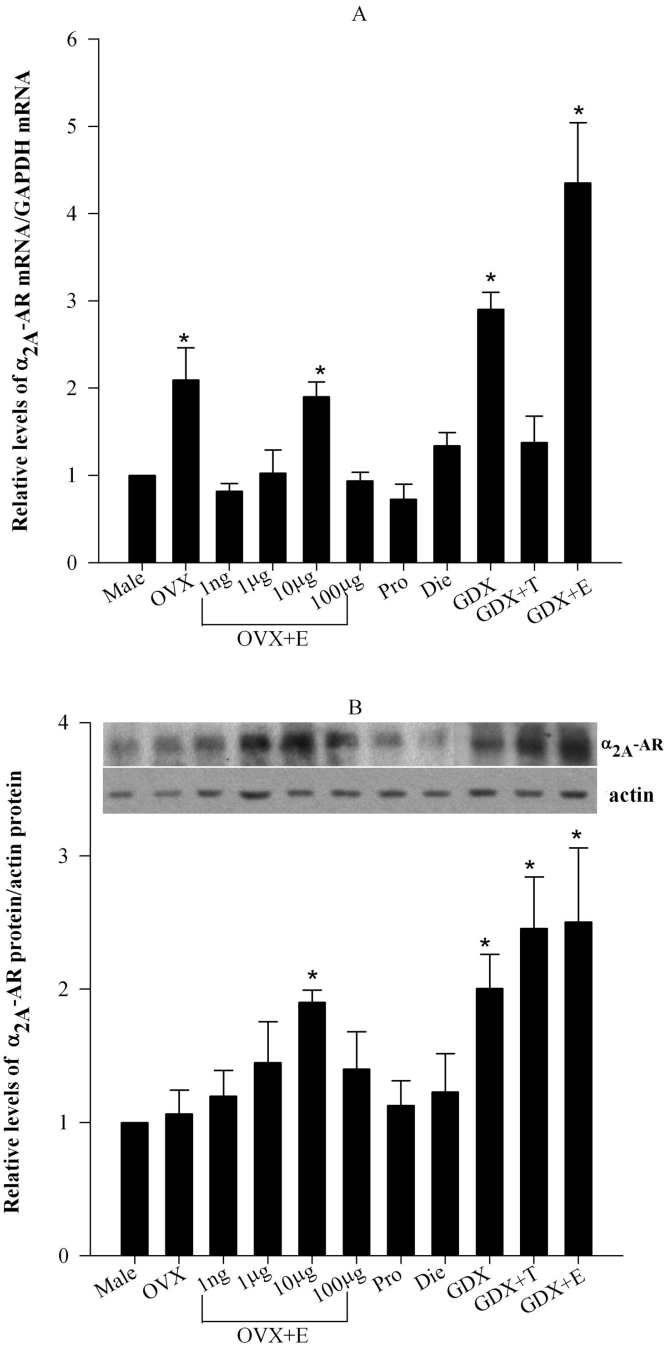


Figure 4. There is no correlation between the expression of α_{2A} -AR and antinociception
A. Levels of α_{2A} -AR mRNA were quantified in the lumbar spinal cord using real-time PCR and normalized by levels of GAPDH mRNA. α_{2A} -AR mRNA levels were significantly higher in OVX, 10 μ g estradiol-treated OVX, GDX and GDX+E groups in comparison to males. However, there was no significant difference between the male and OVX groups treated with 1 ng, 1 μ g and 100 μ g estradiol, proestrous, diestrous and GDX+T groups. **B.** Levels of α_{2A} -AR protein in crude plasma membrane fractions were analyzed in the lumbar spinal cord using optical density and were normalized with β -actin. The top panel shows representative α_{2A} -AR and β -actin immunoblots. The bottom panel represents the

densitometric quantification of α_{2A} -AR normalized to β -actin and compared to the male group. Significant increase in α_{2A} -AR protein levels was noted in the OVX+E (10 μ g), GDX, GDX+T and GDX+E groups. However, there were no significant differences in the OVX, OVX+E (1 ng, 1 μ g and 100 μ g), proestrous and diestrous groups in comparison to the male. * p <0.05