Sex, Pain, and Opioids: Interdependent Influences of Sex and Pain Modality on Dynorphin-Mediated Antinociception in Rats

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ABSTRACT

The role of dynorphin A (1-17; Dyn) and its associated kappa opioid receptor (KOR) in nociception represents a longstanding scientific conundrum: Dyn and KOR (Dyn/KOR) have variously been reported to inhibit, facilitate, or have no effect on pain. We investigated whether interactions between sex and pain type (which are usually ignored) influenced Dyn/KOR-mediated antinociception. Blockade of the spinal Dyn or KOR may result from the presence of nonfunctional (silent) KORs on nociceptive spinal neurons that are responsive to intraplantar formalin (in females) versus intraperitoneal acetic acid (in males). Our findings that spinal Dyn/KOR-mediated antinociception depends on interactions between sex and pain type underscore the importance of using both sexes and multiple pain models when investigating Dyn/KOR antinociception.

Introduction

The opioid dynorphin A (1-17; Dyn) and its principal target, kappa opioid receptor (KOR) (Lord et al., 1977; Chavkin and Goldstein, 1981a; Chavkin and Goldstein, 1981b; Chavkin et al., 1982), have both long been considered to be relevant to nociception. Nevertheless, the role of Dyn and/or KOR (hereafter referred to as Dyn/KOR) in pain processing represents a major and longstanding scientific conundrum. Endogenous Dyn/KOR has been reported to inhibit pain (Medina et al., 1993a; Medina et al., 1993b; Dawson-Basoa and Gintzler, 1996; Ossipov et al., 1996; Tan-No et al., 1996; Dawson-Basoa and Gintzler, 1998; Wu et al., 2002), facilitate pain (Kajander et al., 1990; Xu et al., 2004; Wu et al., 2005), or have no effect on pain (Wu et al., 2002). Similarly, the exogenous administration of KOR agonists have been reported to inhibit nociception (Schmauss and Yaksh, 1984; Nakazawa et al., 1985; Spampinato and Candeletti, 1985; Schmauss, 1987; Yamada et al., 2006; Lawson et al., 2010; Kindler et al., 2011), facilitate nociception (Long et al., 1988; Vanderah et al., 1996; Laughlin et al., 1997), or have no effect on nociception (Stevens and Yaksh, 1986; Kim et al., 2011). There are also numerous reports of discrepancies between antinociceptive effects of Dyn versus KOR (Stevens and Yaksh, 1986; Wu et al., 2002; Lawson et al., 2010). Currently, there is no conceptual framework with which to understand these inconsistent and contradictory findings.

Many of the factors that influence the antinociceptive functionality of spinal Dyn and KOR are sexually dimorphic. Examples include, (1) in females, Dyn/KOR (in addition to μ-opioid receptors; MOR) mediates intrathecal morphine antinociception, whereas in males, it is mediated exclusively by MOR (Liu et al., 2007); (2) endogenous ovarian sex steroids activate spinal Dyn/KOR antinociception (Medina et al., 1993a; Medina et al., 1995; Dawson-Basoa and Gintzler, 1996; Dawson-Basoa and Gintzler, 1998; Lawson et al., 2010), which results, in part, from the disinhibition of lumbar Dyn neurons via the removal of negative opioid and N/OFQ modulation (Gupta et al., 2001); (3) both the density of KOR and its distribution within spinal axon terminals are sex-dependent (Harris et al., 2004); and (4) during proestrus, endogenous estrogen(s) acting through multiple membrane estrogen receptors enhance(s) the propensity of spinal KOR to heterodimerize with MOR, which augments KOR-mediated antinociception (Chakrabarti et al., 2010; Liu et al., 2011a). In view of these sexually dimorphic characteristics of spinal Dyn/KOR-mediated antinociception, it is not surprising that

ABBREVIATIONS: ANOVA, analysis of variance; Dyn, dynorphin 1-17; KOR, kappa opioid receptor; MOR, mu-opioid receptor; α2-NAR, α2-noradrenergic receptor; nor-BNI, nor-binaltorphimine; TBS, tris-buffered-saline; TFL, tail flick latency.
KOR-selective agonists have been reported to have greater antinociceptive potency in proestrus versus diestrus or male rats (Lawson et al., 2010) and in women versus men (Gear et al., 1996a; Gear et al., 1996b; Gear et al., 1999; Gear et al., 2003).

In addition to sex, pain modality also influences the expression of Dyn/KOR-mediated antinociception. For example, in the rat, intrathecal or intracerebroventricular administration of KOR agonists reportedly does not inhibit cutaneous thermal (hotplate, tail flick) responses (Schmauss and Yaksh, 1984; Nakazawa et al., 1985; Schmauss, 1987) but dose dependently inhibits visceral chemical or pressure nociception (Schmauss and Yaksh, 1984; Schmauss, 1987). Pain modality–specific antinociception mediated by KOR has also been documented in humans (Danzebrink et al., 1995).

Previously, the influences on nociception of sex and pain type have been investigated as two independent variables; the relevance of their interaction to nociception has not been as-siduously explored. In this study, we examined the influence of pain modality on the antinociception mediated by spinal Dyn/KOR in both females and males to test the hypothesis that neither sex nor pain modality acts independently of the other. Specifically, we investigated whether different types of pain (chemical/inflammatory or thermal) influenced antinociceptive responsiveness to Dyn/KOR differently in females and males. To focus on the physiologic relevance of Dyn/KOR to nociceptive processing, we studied the nociceptive consequences of endogenously released Dyn. Results revealed that a striking interaction of sex and pain type determined whether endogenously released Dyn and consequent activation of KOR resulted in antinociception.

**Materials and Methods**

**Experimental Animals.** Experiments used Sprague-Dawley rats (Charles River, Kingston, NY; 250–350 g), which were maintained in an approved controlled environment on a 12-hour light/dark cycle. Food and water were available ad libitum. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of SUNY Downstate Medical Center.

**Determination of Stage of Estrous Cycle.** Stage of cycle was determined using histology of vaginal smears. Proestrus was indicated by the predominance of large round nucleated cells. Disruptions of the estrous cycle that could result from surgery did not confound data interpretation, because vaginal smear histology, not predictions that assumed regularity of cycling, was used to define diestrus and proestrus.

**Intrathecal Administration of Drugs.** Yohimbine (Sigma-Aldrich, St. Louis, MO), nor-binaltorphimine (nor-BNI; NIDA), and anti-Dyn antibody (Bachem, Torrance, CA) were administered in 5–10 μl of water. UK14,304 (Tocris, Minneapolis, MN) was administered in 3 μl dimethylsulfoxide over a 60-second period to the subarachnoid space of the lumbar spinal cord via a permanent indwelling intrathecal cannula. Complete delivery was insured by flushing the cannula with an additional 10 μl of saline. The tester was blinded to the nature of the intrathecal treatment (e.g., yohimbine, nor-BNI, anti-Dyn antibody). Thereafter, spinal Dyn release or responsiveness to nociceptive stimuli was determined at various intervals and compared with predrug values. Neither of the vehicles used to dissolve drugs (water, dimethylsulfoxide) altered basal nociceptive thresholds, spinal yohimbine antinociception, or Dyn release.

**In Vivo Perfusion of Spinal Intrathecal Space.** The intrathecal space was perfused (5 μl/minute) using the push-pull method as previously implemented (Liu et al., 2011b). Two PE-10 catheters (8.5 cm length for inflow and 7.0 cm for outflow) were introduced into the spinal subarachnoid space via the atlanto-occipital membrane under sodium pentobarbital anesthesia (40 and 50 mg/kg i.p. for females and males, respectively). The longer of the two cannulas extended to the middle of the lumbar enlargement and the shorter one to the caudal portion of the thoracic spinal cord. The intrathecal space was perfused with Krebs-Ringer buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM NaH₂PO₄, 14 mM glucose, 1.2 mM MgCl₂, 25 mM NaHCO₃, 2.5 mM CaCl₂) warmed to 37°C. The outflow tubing was placed on ice to cool the perfusate. The spinal cord was equilibrated with the perfusion medium for 20 minutes, after which, four 10-minute perfusate samples were collected from each animal for quantification of Dyn release: two before (for basal release) and two after intrathecal drug treatment. After the initial 20-minute equilibration period, the basal rate of spinal Dyn release did not significantly vary over the ensuing 70-minute period of intrathecal perfusion. Therefore, release of Dyn in the presence of drug treatment was compared with the basal release immediately preceding it. We did not determine the effect of stage of estrous cycle on yohimbine regulation of spinal Dyn release, because differences in effects of intrathecal yohimbine on writhing between proestrous and diestrous animals were not observed. Because intrathecal yohimbine enhanced spinal Dyn release in both females and males, confirmation that activation of α₂- noradrenergic receptors (α₂-NArs) inhibited Dyn release was not repeated in males.

**Dyn-Competitive Peptide Enzyme Immunoassay.** The content of Dyn in intrathecal perfusate was quantified using a competitive enzyme immunoassay performed as instructed by the manufacturer (Peninsula Laboratories Inc., San Carlos, CA) and as previously used (Liu et al., 2011b). The anti-Dyn antibody used for immunoassay of perfusate is highly selective for Dyn: it does not recognize dynorphin 1-13, dynorphin 1-8, α-Neoeendorphin, β-endorphin, dynorphin B, or Leu-enkephalin (see Gintzler et al., 2008, for further characterization). Biotinylated-Dyn (6 pg/well; Peninsula Laboratories Inc., CA) was used as tracer. Plates were counted by an Envision 2102 Multilabel Plate Reader (PerkinElmer, Waltham, MA). A standard curve (0.5–32 pg/assay well) in which the value of absorbance was plotted against the logarithm of unlabeled Dyn in the reaction well was generated in each assay. Values of experimental samples were calculated from the standard curve with use of the linear regression function of the Graphpad Prism (v4.00; GraphPad Software, San Diego, CA). All standard and experimental samples were run in duplicate.

**Assessment of Nociceptive Responses.** Writhing was elicited by intraperitoneal injection of 1 ml of a 2% (w/v) solution of acetic acid (Koster et al., 1959). The latency to writhing and the writhing frequency during the ensuing 60 minutes was determined. For formalin-induced paw flinch, formalin (5%, 50 μl) was injected subcutaneously into the plantar surface of the left hindpaw of the rat. Flinching behaviors (rapid shaking of the paw) were quantified as previously described (Sawynok et al., 1998). After injections, flinching was counted during 0–5 minutes (representing phase I) and during 30–35 minutes and 40–45 minutes after injection, the mean of which was used to represent phase II, which manifested an inflammatory component (Tjoelen et al., 1992). Tail flick latency (TFL) to radiant heat was quantified using a Tail Flick Analgesia Meter (IITC, Woodland Hills, CA). Intensity of the radiant heat was adjusted, such that baseline values were 3.5–4.5 seconds. A cutoff of 10 seconds latency prevented any untoward consequences to the tail.

**Immunocytochemistry and Microscopy.** Rats were perfusion-fixed at SUNY Downstate with use of 4% formaldehyde containing 15% v/v saturated picric acid solution. Vertebral columns were shipped overnight to Minnesota for processing, and spinal segments T11-L6 were identified and removed. Tissue was quickly frozen, and serial sections were cut to a nominal thickness of 5–10 μm on a cryostat (Bright Instruments, Huntington, UK), thawed onto Probe-On Plus microscope slides (Fisher Scientific, Minneapolis, MN), and stored at −20°C until use. Heat-induced antigen retrieval was used in these studies to enhance staining for c-Fos: sections were rinsed in distilled water, immersed in citric acid solution (10 mM citric acid
trisodium salt dehydrate and 0.05% Tween-20; pH = 6.0), and autoclaved at 101°C for 30 minutes. The sections were allowed to cool to room temperature, rinsed in TBS (150 mM NaCl and 25 mM tris-HCl; pH = 7.4), and incubated in permeabilization solution (TBS containing 0.2% Triton X-100 and 0.2% Tween-20) for 1 hour at room temperature. The sections were incubated with appropriate mixtures consisting of 1:10,000 dilution of guinea pig anti-preprodynorphin 235-248 (preprodynorphin 235-248 is a cryptic portion of the Dyn precursor that serves as a marker peptide for Dyn-ergic neurons [Arvidsson et al., 1995]), 1:300 dilution of rabbit anti-KOR1 (KT2, [Drake et al., 1996]), 1:1000 dilution of rabbit anti-α2-NAR (Stone et al., 1998), 1:1000 dilution of mouse anti-dopamine beta-hydroxylase (MAB308; EMD Millipore, Billerica, MA), and/or 1:3000 dilution of sheep anti-c-Fos (R&D Systems, Minneapolis, MN). All antibody dilutions were made in blocking buffer (TBS containing 0.1% Tween-20 and 0.2% casein). The sections were washed with two changes of TBS and then with permeabilization solution for 1 hour at room temperature. The sections were incubated for two hours at room temperature with a mixture of 3 μg/ml each of donkey anti-rabbit IgG conjugated to Cy3 (Jackson), donkey anti-guinea pig IgG conjugated to Cy2, and donkey anti-sheep Cy5 in blocking buffer. The sections were washed in three changes of TBS, rinsed in distilled H2O, dehydrated in increasing concentrations of ethanol (50–100%), and cleared in xylene. The slides were mounted with coverslips using DPX Mountant (Fluka, Ronkonkoma, NY). Sections were imaged using an Olympus FV1000 confocal microscope.

Only very occasional neurons labeled for c-fos were observed in animals that were not treated with intraplantar formalin or intraplantar enone acetic acid. We estimated the proportion of c-Fos-labeled cells that expressed KOR, using a total of three male rats and three female rats for each stimulus (intraplantar enone acetic acid and intraplantar formalin). C-Fos-labeled cells that were used for quantitative analysis were taken from the marginal zone or substantia gelatinosa; we analyzed all dorsal horn c-Fos-labeled cells in each section used for these studies. Images that were used for quantitative analysis were obtained with pixel sizes in x-y of ~100 nm and z-steps of 300 nm. The confocal pinhole was set to one Airy unit with use of the automatic setting.

Statistical Analyses. A two-way analysis of variance (ANOVA), followed by Bonferroni’s multiple comparison tests, was used to analyze the treatment and time effects on Dyn release or formalin responsiveness. One-way ANOVA was used to analyze writhing responsiveness and Dyn release in or between groups. Repeated-measures ANOVA was used to analyze treatment effects on writhing. An unpaired t test was used to assess the sex difference of basal release of Dyn. Fisher’s exact test was used to determine whether there were different extents of double-labeling among different neuronal populations.

Results

Regulation of Spinal Dyn Release by α2-NAR in Males and Females. Activation of spinal α2-NARs has been shown to regulate spinal Dyn/KOR antinociception in females (Liu and Gintzler, 1999), but not males (Liu and Gintzler, 2000). To investigate whether this sex difference resulted from differential regulation of spinal Dyn release by α2-NARs, we investigated the effect of intrathecal yohimbine (30 μg), a selective α2-NAR antagonist (Yaksh, 1985; Liu and Gintzler, 1999), on Dyn release. In females, one-way ANOVA within-group analysis revealed a significant treatment effect for yohimbine on spinal Dyn release (F2,10 = 6.52, P = 0.015). Ten to 20 minutes and 30–40 minutes after the intrathecal application of yohimbine, Dyn release increased by 42% and 61%, respectively, above basal (18.68 ± 9.77 pg/10 minutes) (P < 0.05, n = 12 for both time points; Fig. 1A). These results reveal that Dyn release is tonically suppressed by spinal noradrenergic transmission.

Enhancement of Dyn release after blockade of spinal α2-NRs suggests that the highly selective α2-NAR agonist UK14,314 (Buerkle and Yaksh, 1998) would inhibit Dyn release in female rats. Consistent with this inference, we found that during 10–20 minutes and 30–40 minutes after the spinal administration of UK14,314, Dyn release was reduced from 18.27 ± 5.36 to 15.48 ± 4.63 pg/10 minutes and 13.22 ± 5.45 pg/10 minutes, respectively (P < 0.01 for both time points, n = 8; Fig. 1A); intrathecal administration of UK14,314, infact, dose-dependently (0.01–1 μg) inhibited spinal Dyn release (one-way ANOVA within-group analysis: F2,14 = 14.59, P < 0.001; n = 3–8; Fig. 1B). Although the effects of intrathecal UK14,314 (1 μg) significantly differed from the effectsofyohimbine (30 μg) on Dyn release (P < 0.01), their combined intrathecal application resulted in the enhancement of spinal Dyn release that did not significantly differ from that produced by yohimbine alone (P > 0.05; Fig. 1A). The basal rate of spinal Dyn release (i.e., release before drug treatment) did not differ among treatment groups (yohimbine, UK14,314, and their combination; one-way ANOVA: F2,18 = 0.40, P = 0.68; n = 6–8; Fig. 1A). The ability of intrathecal yohimbine and UK14,314 to enhance and inhibit, respectively, spinal Dyn release cross validates that spinal α2-NAR activity is a critical determinant of spinal Dyn-ergic activity. These results indicate that both activation of spinal α2-NARs decreases Dyn release and Dyn release is tonically suppressed by spinal noradrenergic transmission.

Only very occasional neurons labeled for c-fos were observed in animals that were not treated with intraplantar formalin or intraplantar enone acetic acid. We estimated the proportion of c-Fos-labeled cells that expressed KOR, using a total of three male rats and three female rats for each stimulus (intraplantar enone acetic acid and intraplantar formalin). C-Fos-labeled cells that were used for quantitative analysis were taken from the marginal zone or substantia gelatinosa; we analyzed all dorsal horn c-Fos-labeled cells in each section used for these studies. Images that were used for quantitative analysis were obtained with pixel sizes in x-y of ~100 nm and z-steps of 300 nm. The confocal pinhole was set to one Airy unit with use of the automatic setting.

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Intrathecal Yohimbine Attenuates Writhing in Females but Not Males. No differences were observed in latency to writhing frequency during diestrus versus proestrus (latency to writhing: 2.9 ± 0.7 versus 3.4 ± 0.4; writhing frequency: 134 ± 12.8 versus 137 ± 10.1; n = 7 for all). In addition, neither latency to writhing nor writhing frequency differed after intrathecal yohimbine during diestrus versus proestrus (latency to writhing: 6.3 ± 0.8 versus 5.0 ± 0.5; writhing frequency: 68 ± 6.6 versus 61 ± 7.6; n = 8 for all). Therefore, the data from diestrus and proestrus were pooled for subsequent analyses. One-way ANOVA revealed a treatment effect of yohimbine on the latency to writhing (F2,15 = 3.51, P < 0.01; Fig. 2A) and on the number of writhes (F2,15 = 7.13, P < 0.01; Fig. 1B). In contrast to females, the intrathecal application of yohimbine in males had no effect on either the latency or the frequency of writhing (Fig. 2). Because 30 μg yohimbine produced an equivalent increment in spinal Dyn release in females and males, higher intrathecal doses were not tested.

The Effect of Intrathecal Yohimbine Is Mediated by the Spinal Dyn/KOR System. Because clonidine, an agonist at α2-NRs, is well known to be analgesic, it was surprising that yohimbine, an antagonist at α2-NARs, was anti-nociceptive, reducing by more than half the number of writhes
On the basis of the ability of intrathecal yohimbine to release Dyn, we investigated whether Dyn was the mediator of the observed antinociception. Yohimbine’s effects on writhing in female rats were abolished by either the concomitant administration of Dyn antibodies (500 ng; Bachem) or an overnight (18 hour) pretreatment with the KOR blocker nor-BNI (Fig. 2). This, in combination with the absence of any effect of either intrathecal Dyn antibody or nor-BNI alone, indicates that the yohimbine effect was mediated by Dyn release and KOR activation.

Intrathecal Yohimbine, via Dyn/KOR, Reduces Nociception Elicited by Intraplantar Formalin in Males, but Not Females. Because in males, intrathecal yohimbine released spinal Dyn but did not produce antinociception in the writhing test, we investigated whether the increased Dyn release would reduce responsiveness to a different noxious stimulus. Accordingly, we investigated (in males and females) the effects of spinal yohimbine in the formalin paw flinch test.

In males, yohimbine administration significantly reduced formalin-induced paw flinches (2-way ANOVA; F3,19 = 8.39, P < 0.001; Fig. 3) during both phase I and phase II (P < 0.05 for both phases). The effect of yohimbine was abolished after either an 18-hour pretreatment with intrathecal nor-BNI or when yohimbine was administered concomitantly with intrathecal anti-Dyn antibody (Fig. 3). In striking contrast to males, intrathecal yohimbine had no effect in females on formalin-induced paw flinches (Fig. 3).

Coexpression of Dyn and α2c-NAR in Spinal Neurons. Because Dyn release from spinal cord of females and males increased after antagonism of spinal α2-NARs, we hypothesized that α2-NARs were expressed by Dyn neurons. When we double-stained for Dyn and the α2c-NAR, we found that α2c-NAR immunoreactivity was frequently expressed on or in
Dyn-immunoreactive neurons (Fig. 4). This was observed both on neuronal somata (Fig. 4, B-D) and axon varicosities (Fig. 4, E-G). In addition, when we double-stained for Dyn and dopamine β-hydroxylase (dopamine beta-hydroxylase, an enzyme required for synthesis of noradrenaline), we found dopamine beta-hydroxylase–immunoreactive varicosities opposing Dyn-immunoreactive somata (Fig. 5). Similar observations were made in both sexes. Thus, yohimbine's disinhibition of Dyn release appears to be at least in part mediated directly on Dyn-containing neurons.

Absence of Sexually Dimorphic KOR Expression among c-Fos–Labeled Cells after either Intraperitoneal Acetic Acid or Intraplantar Formalin. The ability of sex and pain modality to influence Dyn/KOR-mediated antinociception produced by intrathecal yohimbine suggested that KOR might be differentially expressed by populations of spinal neurons that are activated by intraplantar formalin or intraperitoneal acetic acid in males versus females. To test this hypothesis, we investigated the coexpression of KOR and c-Fos (as a marker of neuronal activation) 1 hour after either intraplantar formalin or intraperitoneal acetic acid. The distribution of c-Fos immunoreactivity after formalin administration was similar to that observed in previous studies (Presley et al., 1990; Todd et al., 1994). Within the superficial dorsal horn, labeled cells were clustered medially in L3-L5, ipsilateral to the injection. C-Fos immunoreactivity was found bilaterally in the superficial dorsal horn after intraperitoneal acetic acid administration and was observed from T11 (the rostral-most segment examined) to L2, with occasional cells found caudal to that (DeLeo et al., 1991). We found that the majority of c-Fos–immunoreactive neurons expressed KOR, regardless of sex or treatment (Fig. 6; male-acetic acid: 36 of 42 c-Fos–immunoreactive neurons; female-acetic acid: 39 of 43 c-Fos–immunoreactive neurons; male-formalin: 81 of 84 c-Fos–immunoreactive neurons; female-formalin: 88 of 91 c-Fos–immunoreactive neurons; no significant differences; Fishers exact tests; n = 3 rats for each sex/treatment group).

Intrathecal Yohimbine Enhances Thermal Nociception in Both Females and Males. With use of radiant heat as the nociceptive stimulus and the TFL assay, intrathecal yohimbine (30 μg) was found to be pronociceptive in both females (F3,18=5.19, P<0.01) and males (F3,18=4.11, P<0.05), although only ~15% decrease in TFL latency for both...
females and males (Fig. 7) was observed. In both sexes, yohimbine-induced nociception was insensitive to intrathecal anti-Dyn antibodies or an 18-hour pretreatment with nor-BNI (F2,9 = 1.22, P = 0.34 for females; F2,9 = 0.23, P = 0.80 for males).

**Discussion**

This study investigated the effects of interactions between sex and pain modality on the antinociception produced by spinally released Dyn and consequent KOR activation. We used a widely used model of visceral pain: writhing elicited by intraperitoneal acetic acid. The antinociception manifest in this pain test was compared with the antinociception observed in two models of somatic pain that used different nociceptive modalities: formalin paw-flinch (chemical/inflammatory) (Tjolsen et al., 1992) and TFL (thermal).

Major findings include the following. First, blockade of spinal α2-NAR induced a comparable increment in Dyn release from the spinal cords of females and males. Second, despite comparable effects on Dyn release, yohimbine substantially reduced visceral nociception (writhing) in females but not males, whereas in males, but not females, it was antinociceptive in the formalin paw-flinch assay. Third, in females and males, spinal Dyn/KOR mediated the yohimbine-induced antinociception, because it was eliminated after either the intrathecal administration of anti-Dyn antibodies or the KOR blocker nor-BNI. It is unlikely that the observed sex/pain type-dependent antinociception induced by yohimbine resulted from differences in yohimbine potency in males and females, because yohimbine produces an equivalent increment in spinal Dyn release, the mediator of the observed antinociception, in both sexes. Thus, the ability of Dyn/KOR to alter nociception is profoundly influenced by not only sex and pain type, but also by their interactions. Fourth, consistent with our behavioral findings, we found that α2c-NARs were expressed by Dyn-ergic neurons. In addition, KOR was expressed by neurons that were opposed by Dyn-expressing terminals, and Dyn-ergic somata were activated by formalin and by acetic acid (see “Anatomical Substrates”).

**Interdependence of the Sex and Pain Modality in the Modulation of Dyn/KOR Antinociception.** Our findings indicate that sex and different types of pain (and/or the body region to which the nociceptive stimuli is applied) profoundly influence whether antinociception mediated via spinal Dyn/KOR is manifest. Our data indicate that descending noradrenergic fibers innervate spinal Dyn neurons expressing α2c-NAR in both females and males. In females, this pathway modulates acetic acid–induced visceral nociception, but not formalin-induced somatic pain, whereas in males, the opposite was found; blockade of α2c-NAR modulated formalin-induced nociception.
somatic nociception, but not acetic acid-induced visceral nociception.

These seemingly paradoxical findings mirror the pervasive contradictory reports concerning Dyn-mediated effects on nociception (Harada et al., 1995; Dawson-Basoa and Gintzler, 1996). Although Dyn has well-described pronociceptive actions, predominantly mediated by N-methyl-D-aspartate receptors (Long et al., 1988); (Vanderah et al., 1996; Laughlin et al., 1997), Dyn also dampens nociceptive input, including that which occurs after nerve injury (Kajander et al., 1990; Draisici et al., 1991). Of interest, although intrathecal Dyn antiserum blocks the increased sensitivity to nociceptive thermal and innocuous mechanical stimuli after spinal nerve injury (Nichols et al., 1997; Malan et al., 2000; Wang et al., 2001), it enhances formalin-induced flinching behaviors (Ossipov et al., 1996), suggesting that the ability of Dyn to enhance or inhibit nociception depends on pain modality. Our finding that Dyn’s actions on nociception differ depending on combined effects of sex and pain modality could underlie, at least in part, the numerous contradictory reports of the effects of Dyn on nociception.

Results from this study highlight the pitfalls of developing models of Dyn/KOR antinociceptive responsiveness based on examination of either females or males, exclusively. If the current study used only males, we could have (incorrectly) concluded that endogenous Dyn/KOR antinociception was manifest in response to somatic chemical/inflammatory but not visceral pain. Alternatively, if this study had used only females, we could have (incorrectly) concluded the reverse. Not only did early conflicting reports of the effects of endogenous Dyn and KOR assess different pain types (e.g., mechanical-paw-withdrawal threshold and thermal paw withdrawal latencies after constrictive or partial ligation-induced neuropathy (Kajander et al., 1990; Xu et al., 2004; Wu et al., 2005)) versus formalin/paw flinch in non-manipulated animals (Ossipov et al., 1996; Tan-No et al., 1996; Wu et al., 2002)), but these reports were also based on studies that used only one sex (males). Thus, the numerous seemingly contradictory reports regarding the role of Dyn/KOR in modulating nociception could have resulted from studying different nociceptive responses in a single sex.

Anatomic Substrates for Interactions between Sex and Pain Modality. Although our data show striking interactions between sex and pain modality that alter Dyn/KOR antinociception, it does not identify the underlying mechanisms. Our data, however, do eliminate several possibilities. First, the interactions between sex and pain type do not appear to be attributable to selective expression of KOR on different populations of spinal nociceptive neurons, because equal proportions of nociceptive neurons coexpressed KOR and c-Fos in males and females, after either formalin or acetic acid administration. Second, few c-Fos–positive neurons coexpressed Dyn in either sex. Thus, it is unlikely that the observed sex- and pain-dependent effects of intrathecal yohimbine are attributable to spinal Dyn neurons that respond differently to different nociceptive stimuli in males versus females. Third, because spinal Dyn was comparably released in response to intrathecal yohimbine in both sexes and α2c-NAR immunoreactivity was expressed by spinal Dyn-ir neurons in both males and females, it is unlikely that the sexually dimorphic pain-specific antinociception produced by yohimbine resulted from sex-specific expression of α2c-NARs on Dyn neurons. Fourth, in both males and females, Dyn-immunoreactive varicosities opposed neurons that expressed c-Fos immunoreactivity, regardless of whether c-Fos was expressed in response to intraperitoneal acetic acid or to intraplantar formalin. Thus, yohimbine’s sex- and pain-specific actions were probably not attributable to Dyn-ergic neurons specifically synapsing onto different populations of nociceptive neurons in males and females.

On the basis of our inability to demonstrate anatomic differences between males and females that would explain the sexually dimorphic influence of pain modality on the expression of Dyn/KOR antinociception, we hypothesize the existence of nonfunctional (silent) KORs, postsynaptic to Dyn terminals, on nociceptive spinal neurons that are responsive to intraplantar formalin in females and intraperitoneal acetic acid in males (Fig. 8). Nonfunctional KORs could result from the presence postsynaptically of uncoupled KORs (e.g., resulting from phosphorylation [Drake et al., 1996; Appleyard et al., 1997; McLaughlin et al., 2004]) or from KORs with altered function (e.g., resulting from glycosylation [Li et al., 2007], alternative coupling, or dimerization [Wang et al., 2005; Chakrabarti et al., 2010; Liu et al., 2011a]). The distribution of silent KORs is likely to be restricted to Dyn release sites, because exogenous KOR agonists (which unlike endogenously released Dyn, can act at nonsynaptic and synaptic KORs) reportedly produce antinociception in the

![Fig. 8. Proposed organization of nociceptive processing in females and males. Form, AA, and Heat represent primary afferents responsive to intraplantar formalin, intraperitoneal acetic acid, and thermal nociceptive stimuli, respectively. In both females and males, Dyn neurons are inhibited by noradrenergic fibers (not shown) that act via α2c-NARs. However, depending on the noxious stimulus, differences in pain modulation exist between males and females. In both sexes, intraplantar formalin activates nociceptive spinal neuron (NsNs; e.g., a nociceptive relay neuron, such as a spinothalamic tract neuron; magenta), which are opposed by Dyn neurons. We propose that, in males, these Dyn-ergic appositions inhibit formalin-induced nociception via KOR, but in females, NsNs express a modified, silent form of KOR (KOR*) that does not respond to Dyn activation. There is an analogous activation of NsNs by AA in males and females (blue), but we propose that the pattern of the regulation of nociceptive responsiveness to AA is reversed: Dyn-ergic appositions inhibit AA-induced nociception in females but not in males, because in males, NsNs express a silent form of KOR (KOR*) postsynaptic to Dyn terminals that does not respond to Dyn activation. In both sexes, NsNs that respond to heat (orange) express α2c-NARs, which inhibit nociception independent of spinal Dyn/KOR.](https://jpet.aspetjournals.org/content/jpet/early/2017/04/07/jpet.2017.203210.x/F7.large.jpg)
writhing test in males (Schmauss and Yaksh, 1984; Schmauss, 1987). An alternative (or additional) explanation to silent KORs is that expression of α2-2-NARs by Dyn neurons is sexually dimorphic, such that in males these neurons innervate formalin-responsive, but not nociceptive nociceptors, whereas in females the reverse pattern prevails.

**Multifaceted Roles of α2-2-NARs, Dyn, and KOR in Nociception.** Numerous reports have demonstrated that intrathecal α2-2-NAR agonists (e.g., clonidine) produce antinociception and antinociceptive synergy with spinal opioids (Ossipov et al., 1990; Buerkle and Yaksh, 1998; Wei and Roerig, 1998; Fairbanks et al., 2002). Consistent with these observations, we found that the intrathecal administration of yohimbine (an α2-2-NAR antagonist) was pronociceptive (independent of spinal Dyn and KOR) in the same test (TPL) previously used to demonstrate Dyn/KOR-mediated antinociception by clonidine. Yohimbine's pronociceptive effect, in combination with the antinociception that we observed, indicates that the tonic activity of spinal α2-2-NARs can decrease or increase sensitivity to noxious stimuli, depending on sex and pain type. These data underscore the complex multifaceted roles of α2-2-NARs, Dyn, and KOR in pain processing and emphasize that nociception and antinociception depend on context and may have mechanisms in common.

**Importance of Disinhibition of Spinal Dyn Release to Nociceptive Processing.** Earlier reports from this laboratory demonstrated that spinal Dyn release was inhibited by pathways using spinal 6-opioid receptors, nociceptin/orphanin FQ, or pituitary adenyl cyclase–activating polypeptide (Gupta et al., 2001; Liu et al., 2011b). Furthermore, blocking inhibition produced by each of the above results in both augmented spinal Dyn release and antinociception (Dawson-Basoa and Gintzler, 1998; Gupta et al., 2001; Liu et al., 2011b). Our present findings that blockade of spinal α2-NARs also enhanced spinal Dyn release underscore that disinhibition of spinal Dyn neurons is commonly used to augment spinal Dyn-ergic tone. The fact that this disinhibition results in antinociception of differential sex-dependence depending on pain modality underscores the central role of Dyn in integrating and coordinating nociceptive inputs.

**α2-2-NARs and Inhibition of Dyn Release.** Spinal noradrenergic transmission could directly modulate activity of spinal Dyn-ergic neurons, because they coexpress α2-2-NARs. The precise mechanism by which activation of α2-2-NARs inhibits Dyn release remains to be established. α2-NARs can functionally couple to Gi and Gs (Eason et al., 1994). Coupling to Gi as well as Gi could mediate inhibition, because Gi activation can inhibit calcium currents, via either the generation of Gαi, (Kamaishi et al., 2004) or by stimulation of phospholipase C, which can result in presynaptic inhibition of transmitter release (Edelbauer et al., 2005).

**Translational Relevance.** Pain modality is known to greatly influence antinociceptive efficacy of opioids in humans (Fillingim, 2002; Koltzenburg et al., 2006; Kindler et al., 2011). There is also evidence in humans of sexual dimorphism in opioid antinociception (Gordon et al., 1995; Sarton et al., 2000; Mogil et al., 2003). These studies resonate with current findings in rats and underscore their translational relevance.

Sexual dimorphism has not been universally observed in studies of nociception in humans (or laboratory animals), which has slowed acceptance of the value of incorporating females into all pain studies. Our findings suggest that these inconsistencies could result from the fact that sexual dimorphism is expressed differently for different kinds of pain. Thus, to fully understand sex differences in pain, it will be necessary to conduct integrated studies that include females and males and concomitantly investigate several different nociceptive stimuli applied to multiple anatomic sites.

Although both sex and pain type are important determinants of Dyn/KOR antinociception, neither variable acts independently of the other. Instead, there is a striking interdependence of sex and pain type that determines the manifestation of the antinociception mediated by Dyn and its receptor. This discovery could provide a context for understanding the inconsistent and contradictory reports regarding the role of Dyn and KOR in nociception in laboratory animals and humans that continues to plague the pain literature and slow inclusion of females in all pain studies.

**Authorship Contributions**

- **Participated in research design:** Liu, Wessendorf, Gintzler.
- **Conducted experiments:** Liu, Schnell, Wessendorf.
- **Performed data analysis:** Liu, Wessendorf, Gintzler.
- **Wrote or contributed to the writing of the manuscript:** Liu, Schnell, Gintzler.

**References**


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