Sex, pain and opioids: inter-dependent influences of sex and pain modality on dynorphin-mediated antinociception in rats

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Running Title Page

a) Running Title: Sex / pain interaction and kappa-opioid antinociception

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c) text pages: 35
   figures: 8
   abstract: 247 words
   introduction: 454 words
   discussion: 1552 words
   references: 73

non-standard abbreviations: Dyn (dynorphin 1-17); KOR (kappa opioid receptor); MOR (mu-opioid receptor); α2-NAR (α2-noradrenergic receptor); nor-BNI (nor-binaltorphimine).
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. Here, we investigated whether interactions between sex and pain type (which are usually ignored) influenced Dyn/KOR-mediated antinociception. Blockade of the spinal α2-noradrenergic receptor (α2-NAR) using yohimbine elicited comparable spinal Dyn release in females and males. Nevertheless, the yohimbine-induced antinociception exhibited sexual dimorphism that depended on the pain test employed: in the intraperitoneal acetic acid-induced writhing test, yohimbine produced antinociception only in females whereas in the intraplantar formalin-induced paw flinch test, antinociception was observed only in males. In females as well as males, both intrathecal Dyn antibodies and spinal KOR blockade eliminated the yohimbine-induced antinociception indicating that Dyn/KOR mediated it. However, despite the conditional nature of spinal Dyn/KOR-mediated yohimbine antinociception, both intraplantar formalin and intraperitoneal acetic acid activated spinal Dyn neurons that expressed α2-NARs. Moreover, Dyn terminals apposed KOR-expressing spinal nociceptive neurons in both sexes. This similar organization suggests that the sexually dimorphic interdependent effects of sex and pain type may result from the presence of nonfunctional (silent) KORs on nociceptive spinal neurons that are responsive to intraplantar formalin (in females) vs. 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 utilizing both sexes as well as 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 vs. 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); (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 KOR-selective agonists have
been reported to have greater antinociceptive potency in proestrus vs. diestrus or male rats
(Lawson et al., 2010) as well as in women vs. 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
assiduously 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 or not different types of pain [chemical/inflammatory, thermal] influenced
antinociceptive responsiveness to Dyn/KOR differently in females vs. males. In order to focus
on the physiological 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 or not endogenously released Dyn and consequent activation of KOR resulted in antinociception.
Materials and Methods

Experimental Animals.

Experiments employed Sprague-Dawley rats (Charles River, Kingston, NY; 250-350 g), which were maintained in an approved controlled environment on a 12 hr 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 since vaginal smear histology and not predictions that assumed regularity of cycling was used to define diestrous and proestrous.

Intrathecal Administration of Drugs

Yohimbine (Sigma, ST Louis MO), nor-binaltorphimine (nor-BNI; NIDA) and anti-Dyn antibody (Bachem, CA) were administered in 5-10 μl of water. UK14,304 (Tocris, Minneapolis, MN) was administered in 3 μl DMSO over a 60 sec 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 as to the nature of the i.t. treatment, e.g., yohimbine, nor-BNI, anti-Dyn antibody, etc. Thereafter, spinal Dyn release or responsiveness to nociceptive stimuli were determined at various intervals and compared with
pre-drug values. Neither of the vehicles used to dissolve drugs (water, DMSO) altered basal nociceptive thresholds, spinal yohimbine antinociception, or Dyn release.

In vivo perfusion of spinal intrathecal space.

The intrathecal space was perfused (5 μl/min) using the push-pull method 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) pre-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 ten-min perfusate samples were collected from each animal for quantification of Dyn release: two prior to (for basal release) and two subsequent to intrathecal drug treatment. After the initial 20 min equilibration period, the basal rate of spinal Dyn release did not significantly vary over the ensuing 70 min 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 since differences in effects of intrathecal yohimbine on writhing between proestrous and diestrous animals were not observed. Since 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., CA) and as previously utilized (Liu et al., 2011b). The anti-Dyn antibody employed for immunoassay of perfusate is highly selective for Dyn: it does not recognize dynorphin 1-13, dynorphin 1-8, α-Neoendorphin, 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 utilized as tracer. Plates were counted by an Envision 2102 Multilabel Plate Reader (Perkin Elmer). A standard curve (0.5-32 pg/assay well) in which the value of absorbance was plotted against the log concentration of unlabeled Dyn in the reaction well was generated in each assay. Values of experimental samples were calculated from the standard curve using the ‘linear regression’ function of the Graphpad Prism software. All standard and experimental samples were run in duplicate.

Assessment of nociceptive responses.

(a) Writhing. Writhing was elicited by intraperitoneal (i.p.) injection of 1 ml of a 2% (v/v) solution of acetic acid (Koster et al., 1959). The latency to writhe as well as the writhing frequency during the ensuing 60 min was determined. (b) 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). Following injections, flinching was counted during 0-5 min (representing phase I) and during 30-35 min and 40-45 min post injection, the average of which was used to represent
phase II, which manifested an inflammatory component (Tjolsen et al., 1992). (c) Tail Flick Latency (TFL). TFL to radiant heat were 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 sec. A cutoff of 10 sec latency prevented any untoward consequences to the tail.

Immunocytochemistry and microscopy

Rats were perfusion-fixed at SUNY Downstate using 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 to 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 used. 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 (135 mM NaCl and 25 mM tris-HCl; pH = 7.4) then incubated in permealization solution (TBS containing 0.2 % Triton X-100 and 0.2 % Tween-20) for one 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-α2c-NAR (Stone et al., 1998), 1:1000 dilution of mouse anti-dopamine beta-hydroxylase (MAB308, EMD Millipore, Billerica, MA) and/or 1:3,000 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 once with permeabilization solution over one 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 i.p. 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 (i.p. 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 about 100 nm and z-steps of 300 nm. The confocal pinhole was set to one Airy unit using 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 within or between groups. Repeated Measures ANOVA was used to analyze treatment effects on TFL. 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). In order to investigate whether or not 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 (F_{2,10}=6.52, p=0.015). Ten to 20 min and 30-40 min following the intrathecal application of yohimbine, Dyn release increased 42% and 61%, respectively, above basal (18.68±9.77 pg/10 min) (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 following blockade of spinal α₂-NARs suggested 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 min and 30-40 min following the spinal administration of UK14,314, Dyn release was reduced from 18.27±5.36 to 15.48±4.63 pg/10 min and 13.22±5.45 pg/10 min, respectively (p<0.01 for both time points, n=8; Fig. 1A); intrathecal administration of UK14,314 in fact dose-dependently (0.01-1 μg) inhibited spinal Dyn release (one-way ANOVA within-group analysis: F_{2,14}=14.59, p<0.001; n=3-8; Fig. 1B). Although the effects of intrathecal UK14,314 (1 μg) significantly differed from the effects of yohimbine (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 prior to 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 not only indicate that activation of spinal α2-NARs decreases Dyn release, but that Dyn release is tonically suppressed by spinal noradrenergic transmission.

The basal rate of Dyn release from spinal cord of males (8.4±0.92 pg/10 min) was significantly lower than that observed in females (18.68±3.26 pg/10 min) (unpaired t-test, p=0.012). However, as was observed in females, the intrathecal application of yohimbine to males significantly increased spinal Dyn release (Fig. 1C; one-way ANOVA within-group analysis: F2,12=6.79, p=0.011; n=5, p<0.05 and p<0.01 for 10-20 min and 30-40 min collection periods, respectively, using Dunnett’s multiple comparison test). Since 30 μg intrathecal yohimbine produced equivalent enhancement in spinal Dyn release in females and males (Fig. 1C), we used this treatment to investigate (without the potential confound of sex-dependent differential Dyn release) the influence of pain type on antinociception mediated by Dyn and KOR in males vs. females.

Intrathecal yohimbine attenuates writhing in females but not males

No differences were observed in latency to writhe or writhing frequency during diestrus vs prooestrus [latency to writhe: 2.9±0.7 (diestrus) vs. 3.4±0.4 (prooestrus); writhing frequency: 134±12.8 (diestrus) vs. 137±10.1 (prooestrus); n=7 for all]. Additionally, neither latency to writhe nor writhing frequency differed following i.t. yohimbine during diestrus vs. prooestrus [latency to
writhe: 6.3±0.8 (diestrus) vs. 5.0±0.5 (proestrus); writhing frequency: 68±6.6 (diestrus) vs. 61±7.6 (proestrus); 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 writhe (F_{5,51}=3.51, p<0.01, Fig. 2A) and on the number of writhes (F_{5,52}=7.13, p<0.001, 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). Since 30 μg yohimbine produced an equivalent increment in spinal Dyn release in females and males, higher i.t. doses were not tested.

*The effect of intrathecal yohimbine is mediated by the spinal Dyn/KOR system.*

Since clonidine, an agonist at α2-NARs, is well known to be analgesic, it was surprising that yohimbine, an antagonist at α2-NARs, was antinociceptive, reducing by more than half the number of writhes (Fig. 2B). Based on the ability of intrathecal yohimbine to release Dyn, we investigated whether or not 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, Torrance, CA) or an overnight (18 h) 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.*

Since in males, intrathecal yohimbine released spinal Dyn but did not produce antinociception in the writhing test, we investigated if 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; F$_{3,19}$=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 following either an 18 h pretreatment with intrathecal nor-BNI or when yohimbine was administered concomitantly with intrathecal anti-Dyn antibody (Fig. 3, left panel). In striking contrast to males, intrathecal yohimbine had no effect in females on formalin-induced paw flinches (Fig. 3, right panel).

Co-expression of Dyn and α$_{2c}$-NAR in spinal neurons.

Since Dyn release from spinal cord of females and males increased after antagonism of spinal α$_{2}$-NARs, we hypothesized that α$_{2}$-NARs were expressed by Dyn neurons. When we double-stained for Dyn and the α$_{2c}$-NAR, we found that α$_{2c}$-NAR-immunoreactivity (-ir) was frequently expressed on or in Dyn-ir 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 (DBH, an enzyme required for synthesis of noradrenaline), we found DBH-ir varicosities apposing Dyn-ir 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 i.p. 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 i.p. acetic acid in males vs. females. To test this hypothesis, we investigated the co-expression of KOR and c-Fos (as a marker of neuronal activation) one hour following either intraplantar formalin or i.p. acetic acid. The distribution of c-Fos-ir 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-ir was found bilaterally in the superficial dorsal horn after i.p. 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-ir neurons expressed KOR, regardless of sex or treatment (Fig. 6; male-acetic acid: 36 of 42 c-Fos-ir neurons; female-acetic acid: 39 of 43 c-Fos-ir neurons; male-formalin: 81 of 84 c-Fos-ir neurons; female-formalin: 88 of 91 c-Fos-ir 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._

Using radiant heat as the nociceptive stimulus and the TFL assay, intrathecal yohimbine (30 μg) was found to be pronociceptive in both females ($F_{3,18}=5.19$, $p<0.01$) and males ($F_{3,18}=4.11$, $p<0.05$), 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 h pretreatment with nor-BNI ($F_{2,9}=1.22$, $p=0.34$ for females and $F_{2,9}=0.23$, $p=0.80$ 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 employed a widely used model of visceral pain: writhing elicited by i.p. acetic acid. The antinociception manifest in this pain test was compared with the antinociception observed in two models of somatic pain that utilized different nociceptive modalities, formalin paw-flinch (chemical/inflammatory) (Tjolsen et al., 1992) and TFL (thermal).

Major findings include the following: (1) Blockade of spinal $\alpha_2$-NAR induced a comparable increment in Dyn release from the spinal cords of females and males. (2) 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. (3) In females and males, spinal Dyn/KOR mediated the yohimbine-induced antinociception since it was eliminated following 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 vs. females since 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. (4) Consistent with our behavioral findings, we found that $\alpha_2c$-NARs were expressed by Dyn-ergic neurons. In addition, KOR was expressed by neurons that were apposed by Dyn-expressing terminals, and Dyn-ergic somata were activated by formalin as well as by acetic acid (see “Anatomical substrates” below).

Inter-dependence of the sex and pain modality in the modulation of Dyn/KOR antinociception.
Our findings indicate that sex as well as different types of pain (and/or the body region to which the nociceptive stimuli is applied) profoundly influence whether or not antinociception mediated via spinal Dyn/KOR is manifest. Our data indicate that descending noradrenergic fibers innervate spinal Dyn neurons expressing $\alpha_2$-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 $\alpha_2$-NAR modulated formalin-induced 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 pro-nociceptive actions, predominantly mediated by NMDA 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; Draisci et al., 1991). Interestingly, whereas intrathecal Dyn antiserum blocks the increased sensitivity to nociceptive thermal and innocuous mechanical stimuli following 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 upon 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 employed 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, had this study employed 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 constriction- or partial ligation-induced neuropathy (Kajander et al., 1990; Xu et al., 2004; Wu et al., 2005) vs 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 utilized 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.

Anatomical substrates for interactions between sex and pain modality.

While 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. (1) The interactions between sex and pain type do not appear to be due to selective expression of KOR on different populations of spinal nociceptive neurons, since equal proportions of nociceptive neurons coexpressed KOR and c-Fos in males and females, after either formalin or acetic acid administration. (2) 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 due to spinal Dyn neurons that respond differently to different nociceptive stimuli in males vs. females. (3) Since spinal Dyn was comparably released in response to intrathecal yohimbine in both sexes, and $\alpha_{2c}$-NAR-ir 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 $\alpha_{2c}$-NARs on Dyn neurons. (4) In both males and females, Dyn-ir varicosities apposed neurons that expressed c-Fos-ir, regardless of
whether c-Fos was expressed in response to i.p. acetic acid or to intraplantar formalin. Thus yohimbine’s sex- and pain-specific actions were probably not due to Dyn-ergic neurons specifically synapsing onto different populations of nociceptive neurons in males and females.

Based on our inability to demonstrate anatomical 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 i.p. acetic acid in males (illustrated in Fig. 8). Nonfunctional KORs could result from the presence post-synaptically 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 (Li et al.; 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 since exogenous KOR agonists (which unlike endogenously released Dyn can act at non-synaptic as well as synaptic KORs) reportedly produce antinociception in the writhing test in males (Schmauss and Yaksh, 1984; Schmauss, 1987). An alternative (or additional) explanation to silent KORs is that expression of α2c-NARs by Dyn neurons is sexually dimorphic such that in males these neurons innervate formalin-responsive, but not acetic acid-responsive neurons, whereas in females the reverse pattern prevails.

*Multifaceted roles of α2-NARs, Dyn, and KOR in nociception.*

Numerous reports have demonstrated that intrathecal α2-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 those
observations, we found that the intrathecal administration of yohimbine (an \( \alpha_2 \)-NAR antagonist), was pronociceptive (independent of spinal Dyn and KOR) in the same test (TFL) previously used to demonstrate Dyn/KOR-mediated antinociception by clonidine. Yohimbine’s pronociceptive effect, in combination with the antinociception we observed, indicates that the tonic activity of spinal \( \alpha_2 \)-NARs can decrease or increase sensitivity to noxious stimuli depending on sex and pain type. These data underscore the complex multifaceted roles of \( \alpha_2 \)-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 utilizing spinal \( \delta \)-opioid receptors, nociceptin/orphanin FQ or pituitary adenylyl cyclase-activating polypeptide (Gupta et al., 2001; Liu et al., 2011b). Furthermore, blocking inhibition produced by each of the above not only results in augmented spinal Dyn release but also antinociception (Dawson-Basoa and Gintzler, 1998; Gupta et al., 2001; Liu et al., 2011b). Our present findings that blockade of spinal \( \alpha_2 \)-NARs also enhanced spinal Dyn release, underscore that disinhibition of spinal Dyn neurons is commonly employed 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.

\( \alpha_2 \)-NARs and inhibition of Dyn release.
Spinal noradrenergic transmission could directly modulate activity of spinal Dyn-ergic neurons since they co-express $\alpha_2$-NARs. The precise mechanism by which activation of $\alpha_2$-NARs inhibits Dyn release remains to be established. $\alpha_2$-NARs can functionally couple to $G_i$ as well as to $G_s$ (Eason et al., 1994). Coupling to $G_s$ as well as $G_i$ could mediate inhibition since $G_s$ activation can inhibit calcium currents, via either the generation of $G_{\beta\gamma}$ (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 anatomical sites.

While both sex and pain-type are important determinants of Dyn/KOR antinociception, neither variable acts independently of the other. Rather, 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 as well as 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, and Gintzler.

Conducted experiments: Liu, Schnell, and Wessendorf.

Performed data analysis: Liu, Wessendorf, and Gintzler.

Wrote or contributed to the writing of the manuscript: Liu, Schnell, Wessendorf, and Gintzler.
References


Legends for Figures

Fig. 1. Alpha-2 adrenergic system negatively regulates spinal Dyn release in females as well as males. The intrathecal space was perfused using the push-pull method (5 μl/min) as described in Materials and Methods. Each spinal perfusate sample (50 μl) was collected over a 10-min period, and the content of Dyn was quantified using a competitive peptide enzyme immunoassay. All drugs were administered via the inflow intrathecal catheter. Data are presented as mean Dyn release (pg/per 10 min) or percent change ± SEM. A, Blockade by yohimbine (YO, 30 μg) and activation by UK14,314 (UK, 1 μg) of spinal α2-NAR stimulated and inhibited, respectively, spinal Dyn release in females (n=6-8). When UK was administered concomitantly with YO, inhibitory effects of UK were eliminated and enhancement of Dyn release continued to be observed. B, Dose-dependent ability of intrathecal α2-NAR agonist UK to inhibit spinal Dyn release in females (n=3-8). C, Blocking spinal α2-NAR with intrathecal YO (30 μg) disinhibited Dyn release from the spinal cord of both females and males; the resulting increase in Dyn release was not sex-dependent (n=8 for females; n=5 for males). Collectively, these data provide cross validation that spinal Dyn release is tonically inhibited by ongoing α2-NAR activity that was not sex dependent.
Fig. 2. Intrathecal $\alpha_2$-NAR blocker yohimbine (YO) is antinociceptive in the writhing test in female but not male rats. Immediately following i.p. injection of acetic acid (1 ml, 2%), the latency for the onset of writhing (A) and the number of writhes (B) were quantified during the ensuing 60 min. Data are presented as mean $\pm$ SEM ($n \geq 6$). *In females, the latency to writhe was substantially increased and number of writhes was decreased by the intrathecal application of YO (30 $\mu$g, applied 30 min before i.p. acetic acid). The intrathecal application of the KOR blocker nor-BNI (BNI, 26 nmol, 18 h pretreatment) or of anti-Dyn antibodies (DynAb, 500 ng, applied together with YO) eliminated both the YO-induced delayed onset and the attenuated frequency of writhing. Intrathecal YO failed to alter writhing latency or frequency in males. For females, $n=9,10,11,10$ for control, YO, BNI+YO, DynAb+YO, respectively. For males, $n=7$ and 6 for control and YO, respectively.

Fig. 3. Intrathecal yohimbine (YO) reduces formalin-induced pain in males but not females. Immediately after the subcutaneous injection of formalin (50 $\mu$l, 5%) into the hind paw, paw flinch was counted during 0-5 min (for phase I), and quantified and averaged during 30-35 min and 40-45 min post injection (for phase II). Data are presented as mean $\pm$ SEM ($n=4-8$). *In males, but not females, intrathecal YO inhibited pain response in both phase I and phase II, which was reversed by spinal KOR blockade, via intrathecal nor-BNI (BNI), as well as by intrathecal administration of a Dyn antibody (DynAb).

Fig. 4. Expression of $\alpha_{2c}$-NAR ($\alpha2c$) by Dyn neurons. A: Overview of Dyn and $\alpha_{2c}$-NAR expression in superficial dorsal horn. The edge of the dorsal horn can be seen in the top right corner of the image. Square box outlines the region shown in B, C and D; arrow points to the varicosity shown in E, F and G. B-D: $\alpha_{2c}$-NAR expression by Dyn somata. Arrows point to sites
where the $\alpha_{2c}$-NAR receptor is expressed within or on Dyn neurons. E-G: Expression of the $\alpha_{2c}$-NAR receptor by a Dyn varicosity. These findings suggest that the $\alpha_{2c}$-NAR can directly modulate both the firing of Dyn neurons and release from Dyn terminals.

Fig. 5. DBH-ir varicosity apposing a Dyn-ir neuron. Projection of 16 optical sections (total distance in z axis=7.5 $\mu$m) of superficial dorsal horn, showing the relationship between a DBH-ir fiber and a Dyn-ir neuron. Inset shows a single optical section in which a DBH-ir varicosity closely apposes the Dyn-ir neuron. Arrows point to the apposition.

Fig. 6. Relationships of KOR and Dyn to neurons expressing c-Fos in females and males, after either i.p. acetic acid (AA) or intraplantar formalin. Each panel shows a single optical section. Neurons expressing c-Fos-ir are marked with an asterisk. C-Fos-ir neurons expressed KOR-ir and were apposed by Dyn-ir varicosities.

Fig. 7. Intrathecal yohimbine (YO) is pronociceptive in the TFL test in both males and females. Basal TFL represents the average of 3 individual determinations prior to treatment. The effect of intrathecal YO on TFL was determined by averaging two individual determinations at 15 and 30 min following the intrathecal administration of YO, YO concomitant with anti-Dyn antibodies (DynAb) or YO following an 18 h pretreatment with nor-BNI (BNI). * Intrathecal YO produced a modest but significant reduction in TFL in both male and female rats, which was not affected by the concomitant intrathecal application of DynAb or the 18 h pretreatment with BNI. Data are presented as mean $\pm$ SEM (n=5-8).

Fig. 8. Proposed organization of nociceptive processing in females and males. Form, AA, Heat, represent primary afferents responsive to intraplantar formalin, i.p. acetic acid and thermal
nociceptive stimuli, respectively. Dyn: spinal neuron expressing dynorphin. KOR: kappa-opioid receptor. KOR*: a modified, inactive form of KOR. α2c: α2c noradrenergic receptor. NsN: nociceptive spinal neuron, e.g., a nociceptive relay neuron such as a spinothalamic tract neuron.

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 NsNs (magenta), which are apposed 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 since, in males, NsNs express a modified form of KOR (KOR*) post-synaptic 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.
Fig. 1

A

Spinal Dyn release (pg)

YO+UK
YO
UK

Time after drug application (min)

10 20 30 40

B

% inhibition of Dyn release

10 15 20 25 30 35

Dose of intrathecal UK (μg)

0.1 1

C

% change of spinal Dyn release

Female, YO
Male, YO

Time after intrathecal YO (min)

10-20 30-40
Fig. 2

A

Control
YO
BNI+YO
DynAb+YO

Writhing latency (sec)

Male
Female

B

Control
YO
BNI+YO
DynAb+YO

Number of writhing

Male
Female

*
Fig. 3

**Male**
- Control
- YO
- BNI+YO
- DynAb+YO

**Female**
- Control
- YO
- BNI+YO

*Number of paw flinches*

**Phase I**
- Male: Control (125), YO (125), BNI+YO (100), DynAb+YO (100)
- Female: Control (100), YO (100), BNI+YO (100)

**Phase II**
- Male: Control (150), YO (150), BNI+YO (150), DynAb+YO (150)
- Female: Control (125), YO (125), BNI+YO (125)
Figure 4

Green = α2c
Red = Dyn

A

E

2 μm

20 μm

B

5 μm

C

D

F

G
Figure 6

A

Female-AA

B

Male-AA

C

Female-formalin

D

Male-formalin

KOR=red
Fos=blue
Dyn=green

* 5 \mu m