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Relationship of spinal dynorphin neurons to delta opioid receptors and estrogen receptor alpha; anatomical basis for ovarian sex steroid opioid antinociception

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d) non-standard abbreviations: DYN (dynorphin); DYN-ir (dynorphin immunoreactivity); ERα (estrogen receptor alpha); ERα-ir (estrogen receptor alpha immunoreactivity); HSP (hormone simulated pregnancy); DOR (delta opioid receptor); DOR-ir (delta opioid receptor immunoreactivity); E2 (17β-estradiol, estrogen); progesterone (P);

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Abstract

Pharmacological and behavioral studies suggest that spinal δ- and κ-opioid antinociceptive systems are functionally associated with ovarian sex steroids. These interactions can be demonstrated specifically during pregnancy or hormone simulated pregnancy (HSP). The analgesia associated with both conditions can be abolished by blockade of either spinal κ-opioid receptors or δ-opioid receptors (DOR). Additionally, both dynorphin (DYN) release (Gupta et al., 2001) and the processing of the DYN precursor (Medina et al., 1995) are significantly increased in the spinal cord during HSP. We undertook the current study in order to determine if DYN, DOR and estrogen receptor α (ERα) share anatomical relationships that permit their direct interaction. Co-expression of DOR or ERα by DYN neurons was assessed using fluorescence immunohistochemistry and a synaptosomal release assay. Findings include (1) ERα and Dyn are co-expressed. Moreover, in spinal cord of HSP animals there were significant increases in the number of DYN immunoreactive (DYN-ir) cells, ERα-ir positive cells, cells double-labeled for DYN-ir and ERα-ir, and the proportion of DYN-ir cells co-expressing ERα. (2) Some varicose fibers in the spinal cord dorsal horn and intermediate gray matter that expressed DYN-ir also expressed DOR-ir. (3) Activation of DORs located on DYN terminals was sufficient to inhibit K+-evoked DYN release. These data define, at least in part, the anatomical substrates that may be relevant to the antinociception of gestation and its hormonal simulation. Furthermore, they provide a framework for understanding sex-based nociception and antinociception and suggest novel strategies for treating pain.
Introduction

Pharmacological and behavioral studies have suggested a functional association between spinal δ- and κ-opioid antinociceptive systems and their modulation by ovarian sex steroids (Boulware et al., 2005). Response thresholds to somatic and visceral noxious stimuli are elevated during gestation (gestational antinociception; GSA) in rats, mice, sows (Gintzler and Bohan, 1990; Iwasaki et al., 1991; Toniolo et al., 1987) and humans (Cogan and Spinnato, 1986). Simulation of the concentration profiles of 17β-estradiol (estrogen; E2) and progesterone (P) found in the blood during pregnancy (i.e. hormone-simulated pregnancy; HSP) also increases thresholds for responsiveness to aversive stimuli (HSP analgesia, HSPA). Moreover, the magnitude, temporal pattern of onset and duration and spinal opioid receptor profile of HSPA are essentially identical to those of physiological pregnancy (Dawson-Basoa and Gintzler, 1993, 1998; Liu and Gintzler, 1999).

In rats, pharmacological blockade of either spinal κ-opioid receptors (KOR) or δ-opioid receptors (DOR) abolishes GSA and HSPA (Dawson-Basoa and Gintzler, 1998; Liu and Gintzler, 2000). This indicates that ovarian sex steroids can induce activation of spinal KOR and DOR analgesic systems and that both of these effects are essential for the manifestation of GSA and HSPA. Functional interactions between spinal DOR and KOR pathways are also indicated by neurochemical analyses. Exposure of non-pregnant ovariectomized animals to pregnancy levels of E2 and P (E2/P) results in a significant increase in spinal cord processing of DYN precursor intermediates (Medina et al., 1995) and in both basal and stimulated rates of lumbar dynorphin release (Gupta et al., 2001). These findings suggest the presence of sex steroid receptors on dynorphin somata and/or terminals. Strikingly, whereas activation of spinal DOR inhibits the stimulated dynorphin release from spinal tissue of control females, DOR activation
facilitates evoked DYN release from spinal tissue of HSP animals suggesting that sex steroid-initiated signaling events can change the responses of spinal DYN neurons to DOR activation.

Although it has been well established that both GSA and HSPA require the participation of DOR, DYN and ovarian sex steroids, the spinal organization supporting interactions among them has not been elucidated. The most parsimonious anatomical relationship among DOR, DYN and ER consistent with their role in GSA and HSPA would be one that allows their direct interaction. Accordingly, we hypothesized that (1) spinal DOR and/or ER are coexpressed by DYN neurons and (2) their expression is affected by ovarian sex steroids. These hypotheses were tested in HSP, a condition that closely mimics the blood concentration profile of E_2/P that occurs during gestation (Bridges, 1984) but avoids the potential confounds of the other aspects of pregnancy, e.g. changes in sensory input due to uterine distension.
Methods

Experimental Animals

Experiments with HSP or control rats employed female Sprague-Dawley rats (Charles River, Kingston, NY; 250-300 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 Animal Care and Use Committees of SUNY Downstate Medical Center.

Induction of HSP. E2/P was administered via the subcutaneous implantation of Silastic tubing filled with either a solution of E2 (in Sesame oil) or crystalline P (Bridges, 1984) as routinely employed by this laboratory (Dawson-Basoa and Gintzler, 1993). Controls consisted of implants containing sesame oil (vehicle for E2) and empty Silastic tubing (as a vehicle control for P). Day one of steroid hormone administration or its vehicle control was initiated at the time of ovariectomy.

Tissue preparation for immunohistochemistry

Rats were deeply anesthetized with a mixture of ketamine (68 mg/kg), xylazine (4.6 mg/kg) and acepromazine (0.9 mg/kg). Rats were perfused through the ascending aorta with 100 ml ice-cold oxygenated calcium-free Tyrode’s buffer (115 mM NaCl, 5 mM KCl, 2 mM MgCl2·6H2O, 400 µM MgSO4·H2O, 3 mM glucose, 25 mM NaHCO3; pH=7.2) followed by 500 ml freshly prepared buffered formaldehyde (4 % w/v formaldehyde, 14 % v/v saturated aqueous picric acid, 75 mM KH2PO4, 85 mM Na2HPO4·7H2O; pH 6.9). After fixation, the entire vertebral column including the pelvis was harvested, placed in cryoprotectant solution (15 mM sucrose,
30 mM K$_2$HPO$_4$, 70 mM Na$_2$HPO$_4$·H$_2$O; pH 7.2) and stored at 4°C until shipped from New York to Minnesota via overnight air courier service.

**Preparation and superfusion of spinal cord synaptosomes**

The spinal vertebral column was sectioned at the intervertebral spaces above vertebrae T-12 and L-1. The lumbar spinal cord contained within this segment (L-1 to L-5; 200-250 mg) was quickly expelled by injecting ice cold saline into the caudal end. Lumbar spinal cord was homogenized in 10 ml of ice-cold 0.32 M sucrose, 5 mM Tris (pH 7.4), using a Teflon/glass homogenizer (15 strokes). Homogenate was centrifuged at 1,000 $g$ for 5 min. The supernatant so obtained was centrifuged at 15,000 $g$ for an additional 20 min. The resulting pellet (P2) was re-suspended in 300 $\mu$l of pre-gassed (95%O2 & 5% CO2) Krebs buffer [containing the protease inhibitors captopril (10 $\mu$M), thiorphan (0.3 $\mu$M), bestatin (10 $\mu$M), and L-leucyl-L-leucine (2 mM)], 200 $\mu$l of which was layered over 100 $\mu$l of Sephadex P-10 slurry (60%) that had been added to a superfusion chamber containing a Whatman GF/B filter at its outlet. The chamber was superfused with Krebs buffer using a Brandel SF-06 Suprafusion apparatus (Gaithersburg, MD). After a 40 min preincubation, basal release was assessed over two 6 min collections, after which 50 mM K$^+$-evoked release was evaluated over 3 min. Subsequently, two additional cycles of release were collected, each separated by a 15 min incubation. The second release cycle was collected in presence of the delta opioid agonist D-Pen$^2$D-Pen$^5$-enkephalin (DPDPE) (1 $\mu$M) after which the conditions for the first cycle were repeated. For all reported data, basal and evoked release in cycles 1 and 3 were indistinguishable.

**Dynorphin radioimmunoassay**

Superfusates containing basal release and evoked release were desalted and concentrated using reverse phase C18 Maxi-Clean cartridges (Alltech, Deerfield, IL). DYN peptide, eluted
with 70% acetonitrile/0.1% trifluoroacetic acid (TFA), was lyophilized to dryness and stored (4°C). Recovery of DYN A (1–17) was quantitative (~95%). DYN content was assessed using a scintillation proximity radioimmunoassay. The standard DYN peptide or the DYN extracted from suprafusate were resuspended in 110 µl of 0.1 M sodium phosphate (0.1% BSA) and incubated with 20 µl (3µg/ml) of purified rabbit anti-DYN antibody (Peninsula Laboratories, San Carlos, CA) for 2 hours at room temperature, after which I^{125} labeled DYN A (Bachem; 10,000 CPM) was added and allowed to incubate for an additional 2 hours at room temperature. 50 µl of anti-rabbit scintillation proximity polyvinyl toluene beads (Amersham Biosciences, Buckinghamshire, UK; 1mg/50 µl in 0.1 M phosphate buffer) was added and incubated overnight at room temperature on a circular shaker. The next day the assay mixture was transferred to a 96-well polystyrene clear plate for counting using a MicroBeta Jet Counter (Perkin Elmer, Wellesley, MA). The minimum detectable concentration of DYN is 1.9 pg, with an ED_{50} of 22 pg.

**Immunohistochemistry**

The L5, L6, S1 and S2 spinal segments were identified and mounted for sectioning. Tissue was quickly frozen and sections were cut at a nominal thickness of 10 µm on a cryostat (Bright Instruments, Huntington, UK), thawed onto Fisherfinest Capillary gap slides, (Fisher Scientific, Pittsburg, PA) and stored at -20°C until used. In order to minimize effects of variations in staining conditions and thus maximize the reliability of our comparisons, spinal cord from two control and two experimental animals were embedded in the same block. This allowed sections from each of the four tissue samples, which could be distinguished by their orientation, to be cut, mounted and processed concomitantly. Sections were rinsed in PBS (140 mM NaCl, 10 mM Na_{2}HPO_{4}, 3 mM KCl, 2 mM KH_{2}PO_{4}; pH = 7.4) and then co-incubated overnight at room temperature with a 1:300 dilution in PBS containing 0.3% Triton X-100 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., 1995b.) One of two other antibodies was added to the anti-preprodynorphin solution: either a 1:1000 dilution of rabbit anti-estrogen receptor alpha (ERα: Millipore) or a 1:1000 dilution of rabbit anti-DOR1 3-17. The sections were washed with three changes of PBS and co-incubated with 3 µg/ml donkey anti-guinea pig IgG conjugated to Cy3 and with 3 µg/ml donkey anti-rabbit IgG conjugated to Cy2 (Jackson Immunoresearch Laboratories, West Grove, PA) in PBS diluent for two hours at room temperature. The sections were washed in three changes of PBS, rinsed in 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).

The specificity of the antisera used in these studies was tested using absorption controls. Controls were performed by adding 10 µg of the peptide against which the antiserum was raised to 1 ml of the diluted antiserum. In all cases, the resulting labeling was substantially reduced or abolished (Supplemental Figures 1 and 2).

Images were collected using either a conventional fluorescence microscope (Olympus BX-50, Tokyo, Japan) or a confocal microscope equipped with laser lines at 488 nm and 543 nm (Olympus Fluoview 1000). Confocal images were collected with a 60X, 1.4 n.a., oil immersion objective. All images are of coronal sections. Digital images were adjusted for publication (e.g. sharpened, resized, merged, and adjusted for brightness and/or contrast) with Photoshop (Adobe, San Jose, CA) or Olympus Fluoview Viewer (Olympus). Control and experimental images were manipulated identically.

Quantitative immunocytochemical studies were performed using four HSP and four control rats; one to six sections per segment per rat were evaluated for these studies. The density of DOR-ir
was evaluated using the ImageJ program. Images were collected using a 40x, 0.85 n.a. objective, filtered using a Fourier short-pass filter (10 pixel maximum), thresholded at an intensity of 112, and the number of above-threshold pixels counted. The numbers of terminals double-labeled for DOR-ir and DYN-ir were also counted. Because automated methods proved unreliable in this case, counting was performed by eye using pairs of images obtained with a 40x, 0.85 n.a. objective.

DYN-ir neurons, ERα-ir neurons and DYN / ERα double-labeled neurons were counted by eye directly from the tissue using a 20x, 0.7 n.a. objective. Since control and HSP tissue were mounted on the same block (see above), it was not possible for the tissue to be evaluated “blindly”. To validate the objectivity of our observations, we used images taken from a subset of the data. Pairs of images were made of the left and right dorsal horns from the same spinal cord (in and adjacent to the lateral substantia gelatinosa); images from the right side were identified with regard to experimental group whereas the identities of the images from the left sides were encoded. The highly significant correlation between the numbers of cells counted on the two sides (Pearson’s r value=0.5526; p=.0051; 24 pairs of images from L6; ERα--ir evaluated) is strong evidence that our awareness of the experimental conditions did not influence results.

Statistical comparisons were made using the Prism software package (GraphPad, San Diego, CA). Unpaired t-tests or Fisher’s exact tests were employed.

Results
**Distributions of DYN and DOR.** Observations were made in spinal segments L5-S2 of 4 control and 4 HSP rats. In control and HSP females, DOR-ir occurred almost entirely as fibers rather than cell somata (Figure 1a). Labeling was densest in the superficial dorsal horn and had a distribution similar to that of small-diameter primary afferent fibers. DOR-ir fibers were also found widely throughout the neck of the dorsal horn and the remainder of the spinal cord gray matter. These findings are consistent with previous descriptions of DOR-ir (Arvidsson et al., 1995a; Dado et al., 1993).

DYN-ir was observed in both cell somata and nerve fibers (Figure 1b). Somata were concentrated in the superficial dorsal horn and in the outermost portions of nucleus proprius but were also occasionally observed in the lateral reticulated area, lamina X, and the intermediate gray of the spinal cord. Fibers were densest in the superficial dorsal horn but were also observed throughout the dorsal horn and the intermediate gray. They were only rarely observed in the ventral horn. Significantly more DYN-ir profiles were present in L5 of HSP rats (14.42 ± 0.95 profiles/section in HSP rats vs. 10.46 ± 0.91 profiles/section in control rats, p=.0042; Figure 2). The same was also found in the L6 segment (11.61 ± 1.01 profiles/section in HSP rats vs. 8.38 ± 0.73 profiles/section in control rats, p=.0122). However, no significant differences were observed for S1 or S2 (S1: 8.91 ± 0.74 HSP vs. 6.96 ± 0.66 control, p=0.0542; S2: 7.22 ± 0.47 HSP vs. 6.78 ± 0.54 control, p=0.5502).

**Coexpression of DYN AND DOR.** The regulation of DYN release by activation of DOR suggested that DORs might be expressed by DYN neurons. Consistent with previous observations, (Arvidsson et al., 1995a; Dado et al., 1993), we did not observe DOR expression by somata. However, DYN-ir terminals frequently (Figure 3), though not always (Figure 4), expressed DOR-ir. Fibers expressing DOR and DYN were observed in the dorsal horn and intermediate gray matter of both HSP and control rats. We did not observe significant
differences in either the numbers of double-labeled terminals in lamina V in L5-S2 (p>0.2, unpaired t-tests) or in the density of DOR-ir in this region (p>.34, unpaired t-tests). The absence of an increase in the number of terminals co-expressing DOR and DYN during HSP suggests that DORs are not present in the neurons in which increased DYN expression occurs. In addition to the co-localization of DYN and DOR, DOR-ir varicosities frequently apposed DYN somata in control and HSP rats (Figure 4). This suggests that regulation of spinal DYN activity can occur via both direct and indirect consequences of DOR activation.

Terminal DORs can modulate DYN release. Expression of DOR by DYN axon terminals would provide a mechanism by which activation of DORs could directly modulate DYN release and is consistent with a previous report from the Gintzler laboratory that DYN release can be modulated by DOR activation (Gupta et al., 2001); evoked release of DYN from minced spinal cord tissue can be dose-dependently inhibited by the DOR agonist DPDPE. However, the minced spinal cord preparation used in those studies contained DYN somata as well as DYN terminals and release could have been modulated at either site. To determine if an action of DPDPE on DORs expressed on terminals would be sufficient to inhibit DYN release, we investigated if stimulated release of DYN from synaptosomes, which are devoid of somata, would still be inhibited by DPDPE. In the absence of DPDPE, basal release of DYN from spinal synaptosomes was 33.3±2.3 pg/6 min. The increment (S-B) in the rate of DYN release evoked by 50 mM K+ was 11.1 pg/6 min (Figure 5, black bar). Notably, in the presence of 1 µM DPDPE (following a three min pretreatment), the K+-evoked increase in the rate of DYN release was reduced to 6.3pg/6 min, a decrement of 4.8 pg (43%: Figure 5, open bar; p=0.03; n=3).

Co-expression of ERα- and DYN. As stated above, spinal KOR activity is essential to GSA and HSPA (Dawson-Basoa and Gintzler, 1998; Liu and Gintzler, 1999; Liu and Gintzler, 2000)
and HSP markedly enhances the processing of spinal DYN precursors, the amount of DYN in spinal tissue (Medina et al., 1993a; Medina et al., 1993b) and DYN release (Gupta et al., 2001). Thus we hypothesize that ovarian steroids affect the function of spinal DYN-ergic neurons. The most parsimonious mechanism would be that DYN neurons express steroid receptors. In order to explore this possibility, we examined expression of ERα by DYN neurons.

In dorsal horn of control female rats we observed a moderate number of ERα-ir structures that appeared to be neuronal nuclei and that were concentrated in the superficial dorsal horn and lateral reticulated area (lamina V) (Figure 6). In HSP females, ERα-ir cells were also found in the superficial dorsal horn and lamina V; in addition they were now lightly distributed throughout the neck of the dorsal horn, the intermediate gray and the ventral horn. The number of ERα-ir nuclei was significantly higher (30%) in L5, L6 and S1 of HSP animals (p<0.02 in all cases; Figure 7). No significant difference was observed in the S2 segment (p>0.4).

DYN-ir neurons frequently co-expressed ERα-ir (Figure 6). The number of DYN-ir neurons that co-expressed ERα-ir was significantly higher in L6 and S1 of HSP animals than in control animals (L6: 3.26 ± 0.32 DYN-ER profiles/section for HSP rats vs. 1.63 ± 0.18 profiles/section for control rats, p<0.0001; S1: 1.78 ± 0.34 DYN-ER profiles/section for HSP rats vs. 0.92 ± 0.17 profiles/section for control rats, p<0.02: Figure 8a). No significant differences were observed for L5 or S2 (p>0.4 in both cases). The proportion of DYN-ir neurons that expressed ERα-ir was also significantly higher in the L6 segment (p=0.03, Fisher’s exact test: Figure 8b) but no others. There was no significant difference in the proportion of ERα-ir profiles that were double-labeled for DYN-ir in any segment (p>0.15 in all cases).
Discussion

GSA is a phenomenon that has been observed in humans as well as other species. Despite the fact that many of the contributing cell-biological substrates mediating it are fairly well understood, the relevant anatomical circuits are not. Current findings shed light on the anatomical organization of this system and allow three general conclusions to be drawn; (1) estrogen receptors (specifically, ERα) are expressed by DYN-ir neurons; (2) DYN-ir varicose fibers in the spinal cord dorsal horn and intermediate gray matter also express DOR-ir; (3) activation of DORs located on DYN varicosities is sufficient to inhibit K⁺-evoked DYN release.

Previous studies have revealed the critical importance of the enhanced activity of the spinal cord DYN/KOR analgesic pathway to both GSA and HSPA (Gupta et al., 2001). However, demonstration of enhanced release of spinal DYN during gestation and HSP and the consequent elevation of nociceptive response thresholds could not distinguish between direct or indirect effects of E₂/P on spinal DYN-ergic neurons. The current finding that ERα is expressed by DYN neurons indicates that spinal DYN-ergic activity can be directly modulated by ERs.

During HSP, in L6, there were significant increases in (1) the number of DYN-ir cells, (2) the number of ERα-ir cells, (3) the number of cells double-labeled for DYN-ir and ERα-ir, and (4) the proportion of DYN-ir cells co-expressing ERα. During HSP, we observed significant increases in the number of ERα-ir cells and the number of cells co-expressing ERα-ir and DYN in L6 and S1. In addition, in L6 we also observed significant increases in the number of DYN-ir cells and in the proportion of DYN-ir cells co-expressing ERα. The increase in the number of DYN-ir cells in L6 (also seen in L5) during HSP is consistent with the increment in DYN content of the lumbar spinal cord observed during gestation (Medina et al., 1993a; Medina et al., 1993b).
and suggests an expansion of DYN-ergic processing of afferent information (both uterine and cutaneous) during these conditions.

The mechanism responsible for the increased co-localization of ER\(\alpha\) and DYN cannot be discerned from current observations. Their increased co-expression could result from de novo expression of DYN by ER\(\alpha\)-bearing neurons. Alternatively, it is possible that ER expression is augmented among all populations of cells, including DYN neurons. Regardless, both mechanisms would have the same functional consequence of increasing the regulatory influence of E\(_2\) on the function of spinal DYN neurons. The ability of physiological levels of E\(_2\)/P to augment ER\(\alpha\) expression during HSP is analogous to observations made in bone where the cellular content and activity of ER\(\alpha\) are regulated by E\(_2\) (Lanyon et al., 2004; Zaman et al., 2006).

Previous studies by one of us (ARG) have found that afferent input from the uterus contributes to GSA: GSA is significantly reduced by sectioning of the hypogastric nerve (Gintzler et al., 1983), which carries afferent input from the rostral uterus to the upper lumbar spinal cord (L1-L3) (Berkley et al., 1993). L6 and S1 receive afferent input from the caudal uterus (including the cervix) via the pelvic nerve (Berkley et al., 1993). Thus our present findings suggest either that the present observations are secondary to changes in the upper lumbar spinal cord, or that that sectioning of the pelvic nerve may also affect GSA and HSPA.

The present studies employed profile counting rather than stereologically unbiased methods to quantify changes in immunohistochemical staining in spinal tissue of HSP rats, due to the difficulty using either the physical or optical disector methods in the sections used in these studies. Profile counting is vulnerable to mis-estimation of cell numbers if the sizes of cells
increase (Reed and Howard, 1998) However, it appears unlikely that this was the case since there were no significant differences (either for DYN-ir or ERα-ir) in the cross-sectional areas of cell profiles (for DYN) or nuclear profiles (for ERα-ir) between control and HSP rats (p>0.57 in both cases). Thus it appears that the present results represent differences in cell numbers as well as differences in cell profiles.

Earlier studies revealed a variety of interactions between opioid systems and ERs. ER regulates synthesis and secretion of the DOR ligand methionine-enkephalin (Amandusson et al., 1999; Low et al., 1989; Romano et al., 1988). ER-mediated regulation of β-endorphin content and secretion is also well established (Lapchak, 1991; Nakano et al., 1991). Additionally, regulation by E2 and ERα of the μ-opioid receptor density in the hypothalamus (Joshi et al., 1993; Mateo et al., 1992) and μ-opioid receptor internalization (Micevych et al., 2003) has been reported. The present demonstration of an association of ERs (ERα) with DYN-ergic neurons in combination with the demonstrated influence of the estrous cycle on spinal KOR (Chang et al., 2000) emphasizes the influence of ovarian sex steroids on endogenous opioids.

Many of the actions of E2 are mediated via protein kinase A. (Auger et al., 2001; Gu and Moss, 1996; Mize and Alper, 2002). Additionally, E2 (via ERα) modulates mitogen-activated protein kinase activation (Zhang et al., 2002) and phosphorylation (activation) of extracellular signal-regulated kinase 1/2 (ERK1/2) is induced by estradiol (Setalo et al., 2002). We speculate that the enhanced release of spinal DYN noted during gestation and HSP (Gupta et al., 2001) could result from activation of ERαs present on DYN-ergic neurons and the initiation of analogous signaling events. Moreover, membrane-bound ERα can activate metabotropic glutamate
receptors (Boulware et al., 2005). Thus, it is possible that estrogen via membrane ERs are able to directly affect the firing of DYN neurons.

The second notable finding is that DYN-ir fibers in the spinal cord dorsal horn and intermediate gray matter co-express DOR-ir, suggesting that DOR agonists modulate DYN release by acting directly on DYN nerve terminals. We (ARG, DG) previously reported that evoked release of DYN from minced spinal cord tissue was dose-dependently inhibited by the DOR agonist DPDPE (Gupta et al., 2001). The minced spinal cord preparation used in those studies contained both DYN somata and DYN terminals, precluding differentiation between these two sites of action. However, somata are not present in the spinal cord synaptosomal preparation used in the current study. Thus, the most parsimonious explanation for ability of DPDPE to inhibit DYN release from spinal synaptosomes would be direct negative modulation of DYN release by terminal DORs. This confirms the functional relevance of our anatomical findings.

Expression of DOR by DYN terminals provides a mechanism by which DOR activation can modulate DYN release selectively at different synapses. The latter could allow pain to be modulated during pregnancy with a great deal of anatomical specificity. Interestingly, modulation of spinal DYN release by DOR reverses from inhibition to activation during HSP (Gupta et al., 2001), indicating its dependence on physiological state.

The present findings cannot directly explain some of the more striking characteristics of HSPA, i.e., the requirement for concomitant activation of both spinal DOR and KOR (Dawson-Basoa and Gintzler, 1998) and the shift from inhibition to enhancement of DOR modulation of spinal DYN release (Gupta et al., 2001). Nevertheless, the spatial relationships we observed among spinal DOR, KOR and ERα justify concluding that they directly interact. We hypothesize that ERα is expressed by neurons co-expressing DYN and DOR; the effects of ER activation on
phosphorylation (see above) and other intracellular signaling events, e.g., altered DOR G protein coupling (Bao et al., 2003), could underlie the qualitative change in effects of DOR activation on DYN release. Co-expression of ERα, DOR and DYN would provide a means by which the effects of estrogens and DOR agonists on antinociceptive circuits could be integrated at DYN cells. This would enable DYN neurons to function as coincidence detectors for the local estrogen milieu and synaptic enkephalins.

Figure 9 integrates current findings and illustrates the circuitry that we propose underlies GSA and HSPA. Present results do not shed light on the extent to which the relationships among DYN-ergic neurons, DOR and ERα and their modulation by ovarian sex steroids are sexually dimorphic. This notwithstanding, demonstration in control animals of co-expression of DYN with DOR or ERα suggest the relevance of these relationships to pain processing in cycling females. The antinociceptive utility of the release of spinal DYN is underscored by the recent demonstration of the dependence on spinal DYN release and KOR activation of i.t. morphine antinociception in female rats (Liu, et al., 2007). Thus, it is tempting to speculate that administration to the lumbosacral cord of appropriate regimens of ovarian sex steroids, alone or in combination with a DOR agonist (which would release DYN after treatment with ovarian sex steroids; Gupta, et al., 2001) could represent a novel pharmacotherapeutic approach to treat chronic pelvic pain in women.
References


Foot Notes

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Legends for Figures

Figure 1: DOR-ir (A) and DYN-ir (B) in the L6 spinal cord dorsal horns of control female rats. A: DOR-ir was observed in varicosities but generally not somata. Inset shows a high magnification view of region outlined by the box. B: DYN-ir was observed in both varicosities and somata. Inset shows DYN-ir somata in the superficial dorsal horn. The distribution of DOR-ir and DYN-ir agrees with previously reports. Bar= 100µm for main images; 25 µm for insets.

Figure 2: Numbers of DYN-ir neurons observed in control female (open bars) and HSP rats (filled bars) at different spinal levels. Significantly more DYN-ir neurons were observed in L5 and L6 of HSP animals (p<.02, unpaired t-tests).

Figure 3: Expression of DOR-ir by DYN-ir terminals in control female (A-C) and HSP rats (D-F). A, D: DYN-ir. B, E: DOR-ir in the same microscopic fields as shown in A and D, respectively. C, F: Merged images in which DYN-ir is red and DOR-ir is green. Arrowheads indicate double-labeling. Bar = 10 µm.

Figure 4: Apposition of DOR-ir terminals onto DYN-ir neurons in L6 of control female (A) and HSP rats (B). Red = DYN-ir; green = DOR-ir. DOR-ir terminals were frequently found in close proximity to DYN-ir neurons in the dorsal horn of both controls and HSP animals (arrowheads). Bar= 5 µm.

Figure 5: Alteration by DPDPE of K⁺-evoked release of DYN from synaptosomes. The size of the increment evoked by 50 mM K⁺ was significantly smaller in the presence of DPDPE than in its absence (*, p= 0.03). Each column represents means ± S.E.M.
Figure 6: Expression and co-expression of ER$\alpha$-ir and DYN-ir in L6 spinal cord dorsal horns of control female and HSP rats. A-C (left column): control female rats. D-F (right column): HSP rats. A, D: DYN-ir. B, E: ER$\alpha$-ir in the same microscopic fields as shown in A and D, respectively. Localization of ER$\alpha$-ir in structures labeled by Hoechst 33258 (inset in B) indicates its expression in cell nuclei. D, F: Merged images showing the relationships between DYN-ir (red) and ER$\alpha$-ir (green). Note expression of ER$\alpha$-ir by some (arrows), but not all (arrowheads), DYN-ir neurons and increased coexpression in HSP animals. Line drawings in C and F show regions from which images were obtained. Bar in F = 25 µm and applies to A-F; bars in line drawings = 1 mm and apply only to those drawings.

Figure 7: Numbers of neurons expressing ER$\alpha$-ir in the dorsal horns of different spinal segments in control females (open bars) and HSP rats (filled bars). Significantly more ER$\alpha$-ir neurons were observed in HSP rats in L5, L6 and S1 (p<.02).

Figure 8: Coexpression of ER$\alpha$-ir and DYN-ir in spinal cord dorsal horn. A: Numbers of neurons coexpressing ER$\alpha$-ir and DYN-ir in the dorsal horns of different spinal segments in control females (open bars) and HSP rats (filled bars). Significantly more ER$\alpha$-ir neurons were observed in HSP rats in L6 and S1 (p<.02; unpaired t-test). B: The proportion of DYN-ir neurons that coexpressed ER$\alpha$-ir in the dorsal horns of different spinal segments in control females (open bars) and HSP rats (filled bars). A significantly higher fraction of DYN-ir neurons were double-labeled for ER$\alpha$-ir in segment L6 in HSP rats (p=.03; Fisher’s exact test).

Figure 9: Illustration of proposed spinal circuitry underlying the analgesic effects of ovarian sex steroids. Since these effects have been shown to be due to spinal release of DYN, we propose...
that the discomfort of pregnancy is ameliorated by DYN inhibition of nociceptive neurons in the dorsal horn, possibly including relay neurons such as spinothalamic tract neurons. A: We envision that relay neurons (RNs) receiving uterine or cutaneous afferents are inhibited by DYN neurons that express ERα. Activation of ERs in pregnancy and HSP increases expression and release of DYN (Medina et al., 1993a; Medina et al., 1993b). Release of DYN can inhibit nociceptive spinal neurons including spinothalamic tract neurons (Willcockson et al., 1986), presumably via activation of KOR. B: Detail of DYN neuronal contact. DYN terminals express DOR; activation of DOR during pregnancy or HSP promotes DYN release (Gupta et al., 2001). DRG: dorsal root ganglion; DOR: delta opioid receptor; KOR: the kappa opioid receptor; DYN: dynorphin.

LEGENDS FOR SUPPLEMENTAL FIGURES

Supplemental Figure 1: Absorption controls for the DYN and DOR antisera. Labeling for DYN and DOR was reduced or abolished in absorption controls. A, B: DYN; C, D: DOR. All images were taken from spinal segment L5 of control animals. A: Normal DYN-ir in dorsal horn. B: Labeling resulting after addition to 1 ml of the diluted antiserum of 10 µg of the peptide against which the DYN antiserum was raised. C: Normal DOR-ir in dorsal horn. D: Labeling resulting after addition to 1 ml of the diluted antiserum of 10 µg of the peptide against which the DOR antiserum was raised. Bar = 50 µm.

Supplemental Figure 2: Absorption control for the ERα antiserum. All images were taken from the dorsal horn of spinal segment L5 of control animals. A, C: ERα labeling. B, D: Nuclear counterstaining with Hoechst 33258. A: Normal labeling with the ERα antiserum. B: Nuclear counterstaining in the same microscopic field as in A. ERα-ir was frequently found associated with Hoechst-labeled nuclei. C: Absorption control. Labeling obtained after addition to 1 ml of...
the diluted antiserum of 10 µg of the peptide against which the ERα antiserum was raised. Labeling for ERα was markedly reduced. D: Nuclear labeling with Hoechst 33258 in the same microscopic field as shown in C. Bar = 50 µm.
FIG 2

Number of cells/section

L5  L6  S1  S2

CTL  HSP

*  **
FIG 5

DYN Release (pg/6min)

<table>
<thead>
<tr>
<th>Basal</th>
<th>Stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL</td>
<td>DPDPE</td>
</tr>
</tbody>
</table>

* Significant difference

# Significant difference between CTL and DPDPE
FIG 7

Number of cells/section

L5  L6  S1  S2

**  ***  *  

CTL  HSP