

# Ovarian Sex Steroid-Dependent Plasticity of Nociceptin/Orphanin FQ and Opioid Modulation of Spinal Dynorphin Release

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## ABSTRACT

Pregnancy and its hormonal simulation via  $17\beta$ -estradiol ( $E_2$ ) and progesterone (P) are associated with spinal opioid antinociception, primarily driven by augmented dynorphin/ $\kappa$ -opioid activity. This study addresses the ovarian sex steroid-activated mechanism(s) that underlie this activation using an ex vivo spinal cord preparation. In lumbar spinal cord obtained from control animals, exogenous  $\kappa$ - or  $\delta$ -opioid agonists (but not  $\mu$ ), as well as nociceptin (orphanin FQ; N/OFQ), dose dependently inhibit the stimulated release of dynorphin. Consistent with these observations, stimulated dynorphin release is enhanced following selective blockade of opioid or N/OFQ receptors, indicating that their endogenous ligands are negative modulators of dynorphin release. In lumbar spinal cord obtained from ovariectomized animals exposed to pregnancy blood levels of  $E_2/P$ , basal and stimulated rates of dynorphin release increase  $\approx 2$ -fold. Moreover, evoked

dynorphin release is no longer negatively modulated by  $\kappa$ - or  $\delta$ -opioid agonists or N/OFQ. Interestingly, in these preparations, release can be facilitated by  $\delta$ -opioid receptor activation, and neither spinal opioid nor N/OFQ receptor blockade enhances evoked dynorphin release. Consistent with these observations, guanosine-5'-O-3-[<sup>35</sup>S]-thio triphosphate binding analyses indicate a reduction in functional N/OFQ receptors. These data indicate that at least part of the  $E_2/P$ -induced augmented activity of lumbar dynorphin neurons results from their disinhibition via the removal of negative opioid and N/OFQ modulation. These results underscore the plasticity of spinal opioid and N/OFQ systems and their dependence on the ovarian sex steroid milieu. Ovarian sex steroid-activated antinociception reveals mechanisms that enable sustained opioid activation without concomitant tolerance formation.

Pregnancy-related antinociception, first demonstrated in rats (Gintzler, 1980), is also manifest in women (Cogan and Spinnato, 1986). It has been demonstrated in rats and sows in response to somatic, as well as visceral, nociceptive stimuli (Toniolo et al., 1987; Kristal et al., 1990; Iwasaki et al., 1991). In rats, the antinociception associated with pregnancy is multifactorial (for review, see Gintzler and Liu, 2000). It involves the pregnancy blood profile of  $17\beta$ -estradiol ( $E_2$ ) and progesterone (P) (Dawson-Basoa and Gintzler, 1993), uterine afferents (hypogastric nerve) (Gintzler et al., 1983), and central components. The latter is comprised principally of descending noradrenergic pathways/spinal  $\alpha_2$ -noradrenergic receptors (Liu and Gintzler, 1999) and spinal  $\kappa$ - $\delta$ -opioid antinociceptive pathways (Medina et al., 1993a,b; Dawson-Basoa and Gintzler, 1996, 1997a, 1998).

Although it has been well established that augmented spinal dynorphin/ $\kappa$ -activity is a prerequisite for the antinociception of gestation and its hormonal simulation (Dawson-Basoa and Gintzler, 1998), the mechanism(s) by which this comes about has not been elucidated. Differentiation between direct activation of spinal dynorphin/ $\kappa$ -analgesic systems versus indirect activation via removal (down-regulation) of pathways that negatively modulate spinal opioid release/responsiveness has remained ambiguous.

Nociceptin (orphanin FQ; N/OFQ) is a recently discovered potential regulator of spinal opioid activity. It is an endogenous heptadecapeptide substrate for the earlier cloned opioid receptor-like 1 receptor (Meunier et al., 1995; Reinscheid et al., 1995). N/OFQ has complex actions (see *Discussion*), among which is the ability to negatively modulate opioid antinociception (Mogil et al., 1996), suggesting that it can act in vivo as an anti-opioid (Harrison and Grandy, 2000).

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**ABBREVIATIONS:**  $E_2$ ,  $17\beta$ -estradiol; P, progesterone; N/OFQ, nociceptin/orphanin FQ; NOR, N/OFQ receptor; HSP, hormone-stimulated pregnancy; DPDPE, D-Pen<sup>2</sup>, D-Pen<sup>5</sup>-enkephalin; TFA, trifluoroacetic acid; RIA, radioimmunoassay; HPLC, high-pressure liquid chromatography; [<sup>35</sup>S]GTP $\gamma$ S, guanosine-5'-O-3-[<sup>35</sup>S]-thiotriphosphate; ANOVA, analysis of variance; SP, substance P.

The anatomical distribution of N/OFQ and its receptor (NOR) manifests considerable overlap with their opioid counterparts (for review, see Harrison and Grandy, 2000). In particular, N/OFQ-like immunoreactive fibers and functional NORs are particularly concentrated in the superficial laminae of the dorsal horn and the area surrounding the central canal. These areas are also rich in dynorphin, enkephalin, and their respective receptors, essential components of gestational antinociception (Dawson-Basoa and Gintzler, 1996, 1997a, 1998).

The anatomical juxtaposition of N/OFQ and opioid pathways in the spinal cord suggests their functional interactivity such that release of spinal opioids could be negatively modulated by endogenous N/OFQ, as was demonstrated in the guinea pig myenteric plexus (Gintzler et al., 1997). Additionally, as was also demonstrated in this preparation, exogenous opioids can negatively modulate the release of their endogenous counterparts (Xu et al., 1989; Gintzler and Xu, 1991) and are thus also potential negative modulators of spinal dynorphin release. Diminution in the negative regulation by either mechanism would enhance spinal opioid (e.g., dynorphin) tone and thus contribute to gestational and ovarian sex steroid-induced antinociception. Accordingly, the influence of N/OFQ, opioids, and the pregnancy blood profile of E<sub>2</sub>/P and interactions thereof on the release of spinal dynorphin A (1–17) were investigated using an ex vivo spinal cord system.

## Materials and Methods

### Experimental Animals

Experiments used female Sprague-Dawley rats (Charles River, Kingston, NY; 250–300 g), which were maintained in an approved controlled environment on a 12-h light/dark cycle. Food and water were available ad libitum. All experimental procedures were reviewed and approved by the Animal Care and Use Committee of the State University of New York, Downstate Medical Center.

### Ovarian Sex Steroid Administration

The pregnancy blood concentration profile of E<sub>2</sub> and P were simulated in nonpregnant, ovariectomized rats (hormone-simulated pregnancy; HSP) via the subcutaneous implantation of Silastic tubing filled with either a solution of E<sub>2</sub> in sesame oil or crystalline P (Bridges, 1984). Controls consisted of implants that contained sesame oil (vehicle for E<sub>2</sub>) and empty Silastic tubing (as a vehicle control for P). Day 1 of steroid hormone administration or its vehicle control was initiated at the time of ovariectomy. Pregnancy-like levels of E<sub>2</sub> and P were achieved by changing the concentration of E<sub>2</sub> in the tubing (10-mm tubing/100 g b.wt.) and by altering the number of 45-mm P implants on days 5, 15, and 19 (see Bridges, 1984; Bridges and Ronsheim, 1987, for details of implantation procedure and comparison with steroid plasma levels of physiological gestation).

### Spinal Tissue Preparation

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, minced using a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co., Gomshall, Surrey, UK; 0.3-mm thickness), placed into a chamber (0.35-ml), and superfused (Brandel Superfusion System, Gaithersburg, MD). The Krebs' solution used for superfusion contained 118 mM NaCl, 4.7 mM KCl, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 1.2 mM MgCl<sub>2</sub>, 2.5 mM CaCl<sub>2</sub>, 11.1 mM dextrose, and gelatin (saturated with 4 g/l), and was gassed with a 95% O<sub>2</sub>/5% CO<sub>2</sub> gas mixture. Additionally the Krebs superfusate used to assess basal and stimulated

dynorphin release contained the protease inhibitors captopril (10 mM), thiorphan (0.3 mM), bestatin (10 mM), and L-leucyl-L-leucine (2 mM) to protect peptides against the degradation resulting from the actions of the tissue proteases.

### Superfusion Paradigm

After an initial equilibration period (20 min), the superfusion buffer was switched to one containing various concentrations of D-Pen<sup>2</sup>,D-Pen<sup>5</sup>-enkephalin (DPDPE;  $\delta$ -opioid receptor agonist), U50,488H (0.1, 10, 100, 1000 nM;  $\kappa$ -opioid receptor agonist), or N/OFQ (0, 1, 10, 100 nM). Responses to N/OFQ were determined with or without blockade of either opioid receptors (via naloxone, 1  $\mu$ M) or NOR (via compound 15, 10  $\mu$ M, a derivative of the NOR antagonist J-113397 that does not contain a hydroxymethyl group on the piperidine ring) (Kawamoto et al., 1999). The effect of naloxone or compound 15, alone, on basal and evoked release was separately determined. The basal release of dynorphin A was determined by quantification of the dynorphin content of spinal superfusate obtained over a period of 18 min (6 ml). The magnitude of stimulated dynorphin release was determined by quantification of the rate of dynorphin release into spinal superfusate that contained high potassium (50 mM K<sup>+</sup>; the content of sodium was proportionally reduced to maintain osmolarity). High K<sup>+</sup>-evoked release of dynorphin was determined over a 9-min period (3 ml). Basal and stimulated superfusates were collected into pre-chilled tubes on ice.

Superfusate containing basal release and evoked release were desalted and concentrated using reverse phase C<sub>18</sub> cartridges (Sep-Pak; Waters Corp., Milford, MA). Dynorphin peptide eluted with 70% acetonitrile/0.1% trifluoroacetic acid (TFA) was lyophilized to dryness and stored (4°C). Recovery of dynorphin A (1–17) was quantitative (>95%).

### Radioimmunoassay (RIA)

**Iodination of Dynorphin.** [<sup>125</sup>I]Dynorphin A (1–17) was generated by the chloramine T procedure. Briefly, 1  $\mu$ g of dynorphin A was sequentially mixed with 5  $\mu$ l of Na[<sup>125</sup>I] (0.5 mCi; Amersham Pharmacia Biotech, Arlington Heights, IL) and 25  $\mu$ l of chloramine T (25  $\mu$ g). [<sup>125</sup>I]Dynorphin A was separated from nonpeptide bound radioactivity, as well as from radioiodinated dynorphin fragments using C<sub>18</sub> Sep-Pak cartridges eluted sequentially with 30 and 70% acetonitrile/0.1% TFA. The 70% acetonitrile eluate (containing ~70% of the total radioactivity) was lyophilized to dryness, resuspended in 0.1% TFA containing 2% acetonitrile, and used as radioactive tracer for the RIA.

**Dynorphin A (1–17) Quantification.** Dynorphin A (1–17) was quantified in spinal superfusate using an RIA that used an antibody (1:10,000) highly specific for this peptide (Peninsula Laboratories, Belmont, CA). A standard curve (1.0–250 pg/assay tube) in which the percentage of inhibition of binding was plotted against the log of unlabeled dynorphin A in the reaction tube was generated in each assay. Bovine serum albumin (0.1%) was included in the assay buffer to minimize nonspecific adherence to the tube surface. After a 2-h incubation at room temperature, radiolabeled dynorphin A (10,000 cpm) was added, and the reaction mixture was incubated overnight (4°C). Antibody-bound radioactivity was separated from unbound tracer by the addition of dextran-coated charcoal (4.5 g of activated charcoal, 0.3 g of dextran, and 15 ml of horse serum in 100 ml of 100 mM sodium phosphate buffer) followed by centrifugation (3,200g at 4°C). The (antibody-bound) radioactivity remaining in the supernatant was quantified using a gamma counter (CliniGamma; Wallac, Inc., Gaithersburg, MD). Values of experimental samples were calculated from the standard curve using Ultroterm software (Wallac, Inc.). The minimum detectable concentration ranged from 1.95 to 3.9 pg/assay tube, which produced ~20% inhibition of maximum binding. A 50% reduction in binding was produced by 11 to 12 pg/assay tube. Aliquots of lyophilized superfusion buffer that had been processed identically to those utilized in release experiments did not

produce any appreciable inhibition of binding. Peptide concentrations were derived from RIA analyses of superfusate that produced between 20 and 75% inhibition of binding, the linear and sensitive portion of the standard curve. All standard and experimental samples were run in triplicate.

The chemical identity of dynorphin-like immunoreactivity was analyzed by combining high-pressure liquid chromatography fractionation (HPLC) with RIA detection. Spinal superfusate (from four lumbar spinal preparations) were collected under basal and stimulated conditions. The dynorphin A peptide contained therein was desalted and concentrated using reverse phase C<sub>18</sub> Sep-Pak cartridges as described above. The 70% acetonitrile-0.1% TFA eluate was lyophilized to dryness, resuspended in 200  $\mu$ l of 2% acetonitrile-0.1% TFA, and centrifuged (500g for 5 min). Fractionation by HPLC was accomplished by applying supernatant (100  $\mu$ l) or the same volume of standard peptide onto a 15-cm C<sub>18</sub> column (5- $\mu$ m Novapak, Waters Associates, Framingham, MA). The column was eluted at a flow rate of 1 ml/min with a mobile phase containing 0.08% TFA throughout and a linear gradient of acetonitrile ranging from 4% at the start to 70% at 28.6 min. These chromatographic conditions permit authentic dynorphin A (1–17) to be separated from other opioid peptides (methionine- and leucine-enkephalin,  $\alpha$ -endorphin,  $\beta$ -endorphin,  $\gamma$ -endorphin, *N*-acetylated  $\beta$ -endorphin). HPLC eluates (1-ml fractions) were lyophilized to dryness, and the content of dynorphin A (1–17) was determined by RIA as described above. Approximately 83% of the dynorphin-like immunoreactivity that was contained in spinal tissue superfusate had a retention time comparable to that of standard dynorphin A (1–17). The data shown have not been corrected for recovery. Krebs' buffer (6 or 3 ml corresponding to basal and evoked releases, respectively) not exposed to tissue but processed as described for tissue superfusate did not contain detectable levels of dynorphin A-like immunoreactivity.

#### Quantification of Spinal Content of N/OFQ

Lumbar content of N/OFQ was quantified using a procedure originally developed for plasma adrenocorticotropin (Rees et al., 1971) using an antibody highly selective for the carboxyl terminus of N/OFQ (1–17). This antibody exhibits no cross-reactivity with N/OFQ (1–11), dynorphins, or endorphins (Quigley et al., 1998). Briefly, cervical, thoracic, and lumbar spinal regions were homogenized (50 mg/ml; 4°C) in buffer containing 10% acetic acid, 0.5% bovine serum albumin, and 3 mM phenylmethylsulfonyl fluoride. Aliquots of the 3,000g supernatant were lyophilized to dryness. A standard curve for N/OFQ (1, 1.95, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250 pg/assay tube, dissolved in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1% bovine serum albumin and 0.5%  $\beta$ -mercaptoethanol) was generated with each RIA. Tracer consisted of Tyr<sup>14</sup> N/OFQ (1–17) (10,000 cpm; Phoenix Pharmaceuticals, Mountain View, CA) that had been iodinated using the chloramine T procedure as described above for the generation of iodinated dynorphin A (1–17). N/OFQ antiserum (courtesy of Dr. David Grandy) was used at 1:5,000 dilution. Antibody-bound radioactivity was separated from unbound tracer and quantified as described for dynorphin.

#### GTP $\gamma$ S Binding

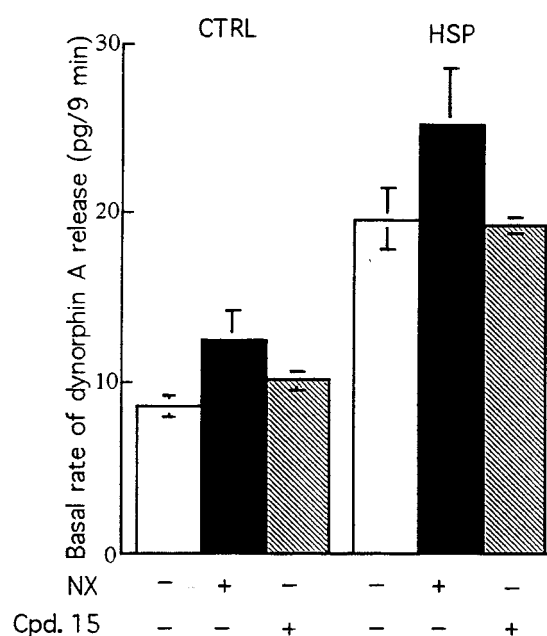
**Membrane Preparation.** Lumbar spinal cord membranes were prepared in 15 volumes (w/v) of ice-cold 0.32 M sucrose as previously described (Narita et al., 1999). Briefly, supernatants resulting from an initial 1,000g (10 min) spin were centrifuged at 20,000g (20 min), the resulting pellets were resuspended in 15 volumes of 50 mM Tris-HCl (pH 7.4), and recentrifuged at 20,000g (20 min). Final pellets were resuspended in 15 volumes of assay buffer [50 mM Tris-HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, 1 mM EGTA, and 100 mM NaCl], aliquoted, and stored at -70°C until further use. The protein contents of membrane preparations were determined using the Bio-Rad protein assay kit (Hercules, CA).

**[<sup>35</sup>S]GTP $\gamma$ S Binding Assay.** The reaction was initiated by the addition of a suspension of spinal lumbar membranes (10  $\mu$ g/assay tube) into the assay buffer (see above) containing various concentrations of N/OFQ (0.1–10,000 nM), 100  $\mu$ M guanosine diphosphate (Sigma, St. Louis, MO), and 50 pM [<sup>35</sup>S]GTP $\gamma$ S (PerkinElmer Life Science Products, Boston, MA) (1 ml total assay volume; 25°C for 2 h). The reaction was terminated by the addition of 5 ml of ice-cold 50 mM Tris-HCl (pH 7.4) and filtration through Whatman GF/B glass filters (Brandel, Gaithersburg, MD) previously soaked in ice-cold 50 mM Tris-HCl containing 5 mM MgCl<sub>2</sub> (pH 7.4). Twice-washed filters were dried using an infrared lamp and transferred to scintillation vials containing 5 ml of scintillation cocktail (Ecoscint H; National Diagnostics, Atlanta, GA). Radioactivity was determined with a liquid scintillation counter (LKB model 1209 Rackbeta; Wallac, Inc.). [<sup>35</sup>S]GTP $\gamma$ S binding in the presence of 10  $\mu$ M unlabeled GTP $\gamma$ S was determined in every assay and defined as nonspecific binding. For the pA<sub>2</sub> determination of the N/OFQ antagonist used, compound 15, N/OFQ dose response for the stimulation of [<sup>35</sup>S]GTP $\gamma$ S binding was determined in its absence and presence (10, 30, and 100 nM) using membranes derived from human NOR-containing Chinese hamster ovary cells. pA<sub>2</sub> values were determined by Schild analysis. Compound 15 was found to be a competitive antagonist that produced a parallel shift in the N/OFQ dose-response curve for stimulation of [<sup>35</sup>S]GTP $\gamma$ S binding (data not shown). The pA<sub>2</sub> value was determined to be 8.48  $\pm$  0.04 ( $K_e$  = 3.44  $\pm$  0.65 nM; slope = 1.08  $\pm$  0.04).

## Results

**Influence of Endogenous Opioids, Exogenous N/OFQ, and Hormonal Milieu on Basal Dynorphin Release from Spinal Tissue.** The effects of spinal opioid receptor blockade, N/OFQ, and ovarian sex steroid treatment on the basal rate of dynorphin release were analyzed using a three-way ANOVA. Main effects of hormone treatment and opioid receptor blockade were observed ( $F_{1,60}$  = 47.75 and  $F_{1,60}$  = 8.63, respectively,  $p \leq 0.005$ ). There was no naloxone by hormone interaction ( $F_{1,60}$  = .991;  $p > 0.05$ ). These results are illustrated in Fig. 1. In lumbar spinal cord obtained from HSP animals (day 19), the basal rate of lumbar dynorphin release increased from 8.7  $\pm$  0.6 to 19.6  $\pm$  1.8 pg/9 min (Fig. 1;  $p < 0.02$ ,  $n = 6$ ). The basal rate of dynorphin release from lumbar tissue obtained from both control and steroid-treated animals was also increased following *in vitro* blockade of opioid receptors with naloxone (1  $\mu$ M). In control preparations, the rate of basal release increased from 8.7  $\pm$  0.6 to 12.5  $\pm$  1.7 pg/9 min following pretreatment with naloxone (1  $\mu$ M). Similarly, in lumbar tissue obtained from steroid-treated animals, opioid receptor blockade increased the basal rate of dynorphin release from 19.6  $\pm$  1.8 to 25.1  $\pm$  3.4 pg/9 min. Basal release from lumbar tissue obtained from either control or HSP animals was not altered by N/OFQ (1–100 nM) or the N/OFQ antagonist compound 15 (10  $\mu$ M).

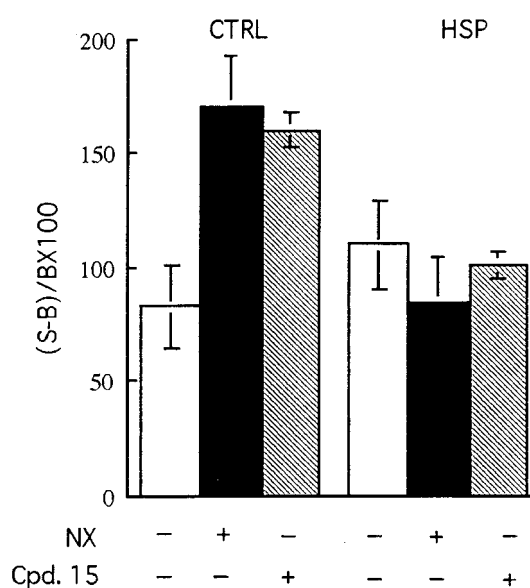
**Influence of Endogenous Opioids, Exogenous N/OFQ, and Hormonal Milieu on High K<sup>+</sup>-Evoked Dynorphin Release from Spinal Tissue.** A three-way ANOVA was also utilized to assess effects of hormone treatment, opioid receptor blockade, and N/OFQ on the fractional rise above the basal rate of dynorphin release elicited by high K<sup>+</sup> (50 mM). Main effects of hormone treatment and N/OFQ (1–100 nM) were observed ( $F_{1,60}$  = 5.727 and  $F_{3,60}$  = 3.952, respectively;  $p \leq 0.02$  for both comparisons). ANOVA also revealed hormone by N/OFQ and hormone by naloxone interactions ( $F_{3,60}$  = 3.965 and  $F_{1,60}$  = 11.154, respectively;  $p \leq 0.01$  for both comparisons). Results are illustrated in Fig. 2.



**Fig. 1.** Effect of pregnancy levels of  $E_2/P$ , opioid, and N/OFQ receptor blockade and interactions thereof on basal dynorphin release from spinal tissue. Lumbar spinal tissue was obtained from untreated and  $E_2/P$ -treated animals and processed as described under *Materials and Methods*. Eluates (collected over 18 min) were desalted, lyophilized, and assayed by RIA for dynorphin immunoreactivity. Each bar represents the mean  $\pm$  S.E.M. obtained from a minimum of five experiments. Blockade of opioid (but not N/OFQ) receptors increased the basal rate of dynorphin release from both preparations, suggesting it to be negatively modulated by endogenous opioids. Basal release was also augmented in spinal tissue obtained from HSP animals providing a biochemical basis for the  $\kappa$ -mediated antinociception observed in these animals. NX, naloxone; Cpd. 15, compound 15; CTRL, control.

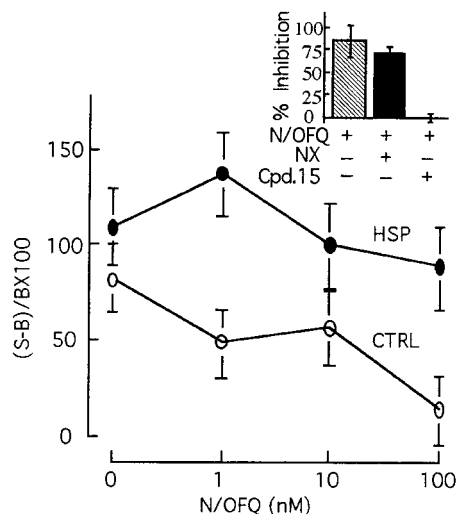
High  $K^+$  (50 mM) significantly increased the rate of dynorphin release from lumbar spinal tissue obtained from control or HSP animals [ $t(5) = -6.80$  and  $t(4) = -3.72$ ,  $p \leq 0.02$  for both comparisons]. However, the magnitude of this effect was dependent upon the hormonal state of the animal. In lumbar spinal tissue obtained from ovarian sex steroid-treated rats, the percent rise above basal elicited by high  $K^+$  did not change (Fig. 2), but the increment (picograms) increased from  $7.1 \pm 1.1$  to  $18.5 \pm 5.5$  pg/9 min ( $\sim 160\%$ ;  $t(9) = 2.99$ ;  $p < 0.05$ ). Figure 2 also illustrates that the in vivo hormonal milieu also influenced the effect of opioid receptor blockade on stimulated dynorphin release, in contrast to observations of basal release. In lumbar spinal tissue obtained from control animals, the  $K^+$ -evoked fractional rise above basal was significantly augmented ( $\sim 2$ -fold) following treatment with naloxone ( $82 \pm 18\%$  versus  $170 \pm 22\%$ ;  $t(8) = 5.57$ ,  $p < 0.001$ ), but this facilitative effect of naloxone was not manifest in spinal lumbar tissue obtained from HSP animals [ $t(8) = 0.712$ ,  $p > 0.4$ ]. Comparable effects on evoked dynorphin release from control versus hormone-treated tissue were observed following treatment with the N/OFQ antagonist compound 15 (Fig. 2). Pretreatment of lumbar tissue obtained from placebo-treated, but not HSP, animals with compound 15 augmented ( $\sim 2$ -fold) high potassium-evoked release [ $82 \pm 18\%$  versus  $160 \pm 7.4\%$ ;  $t(8) = -6.413$ ;  $p < 0.001$ ], consistent with the effects or lack thereof of exogenous N/OFQ in control and steroid-treated preparations, respectively (see below).

Dependence on hormonal milieu was also observed for the negative modulation by N/OFQ of the fractional rise above



**Fig. 2.** Pregnancy levels of  $E_2/P$  abolish the facilitation of  $K^+$ -stimulated dynorphin release produced by blockade of NORs or opioid receptors. Lumbar spinal tissue, obtained from control or  $E_2/P$ -treated animals, was processed, superfusate collected, and its content of dynorphin determined by RIA as described under *Materials and Methods*.  $K^+$ -stimulated release of dynorphin was obtained in the absence or presence of either naloxone ( $1 \mu M$ ) or compound 15 ( $10 \mu M$ ). Each bar represents the mean  $\pm$  S.E.M. percent increase obtained from a minimum of five experiments. NX, naloxone; Cpd. 15, compound 15; CTRL, control; S, stimulated; B, basal.

basal dynorphin release elicited by high  $K^+$  (50 mM; Fig. 3).  $K^+$ -stimulated dynorphin release was dose dependently inhibited by N/OFQ (1–100 nM;  $p < 0.02$ ). An inhibition of  $\sim 85\%$  could be obtained with the highest concentration used. In contrast, in lumbar spinal tissue obtained from HSP ani-



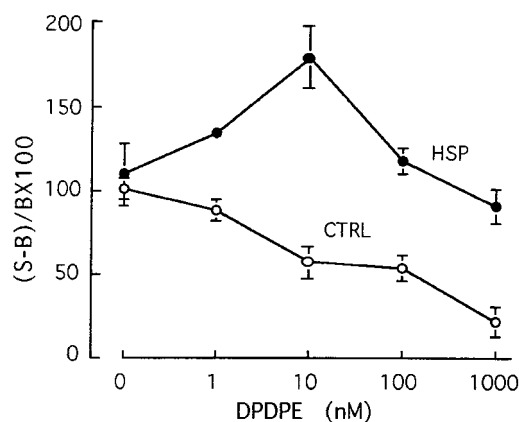
**Fig. 3.** Pregnancy levels of  $E_2/P$  abolish the ability of N/OFQ to dose dependently inhibit the stimulated release of spinal dynorphin. Lumbar spinal tissue was processed, superfusate collected, and its content of dynorphin determined by RIA as described under *Materials and Methods*. Basal and  $K^+$ -stimulated release of dynorphin, in the absence or presence of the indicated concentration of N/OFQ, was determined in spinal tissue obtained from control (○) or  $E_2/P$ -treated animals (●). Each point represents the mean  $\pm$  S.E.M. percent increase obtained from five to six experiments. Inset demonstrates that blockade of NORs via compound 15 ( $10 \mu M$ ) abolishes the  $\sim 85\%$  N/OFQ inhibition of dynorphin release. In contrast, blockade of opioid receptors via naloxone ( $1 \mu M$ ) is without effect. NX, naloxone; Cpd. 15, compound 15; CTRL, control; S, stimulated; B, basal.

mals, no significant inhibition of the rate of stimulated dynorphin release was observed at any N/OFQ concentration tested (1–100 nM). Lack of regulation of lumbar dynorphin release by endogenous N/OFQ in steroid-treated animals does not result from perturbations in lumbar N/OFQ content, since treatment of ovariectomized rats with pregnancy levels of  $E_2/P$  failed to alter the lumbar spinal content of this peptide ( $6.82 \pm 0.44$  versus  $6.44 \pm 0.63$  pg/mg wet weight of tissue;  $n = 3$ ,  $p > 0.2$ ).

The inset to Fig. 3 illustrates the receptor selectivity of the N/OFQ modulation of dynorphin release. As was expected, the ability of N/OFQ to inhibit stimulated dynorphin release from control lumbar tissue was not altered by opioid receptor blockade, i.e., there was no naloxone by N/OFQ interaction ( $F_{3,60} = 1.009$ ,  $p > 0.3$ ). However, the substantial inhibition of evoked dynorphin release by 100 nM N/OFQ was abolished following its coadministration with the selective N/OFQ antagonist, compound 15 (10  $\mu$ M). Thus, the ability of compound 15 to essentially abolish the inhibitory effects of exogenous N/OFQ on stimulated dynorphin release suggests its mediation via lumbar NORs.

**Comparison of Dynorphin Release from Untreated and Vehicle-Treated Animals.** A two-way ANOVA was used to compare the rates of basal and  $K^+$ -evoked dynorphin release elicited in the presence of varying concentrations of N/OFQ (1–100 nM) among untreated and vehicle-treated animals (ovariectomized animals receiving sesame oil and empty Silastic implants). The magnitude of the basal and stimulated rates of dynorphin release, as well as the slope of the N/OFQ concentration-inhibition curve, did not differ among preparations obtained from either control group ( $p > 0.4$ ; data not shown).

**Influence of Hormonal State on  $\delta$ - and  $\kappa$ -Opioid Receptor-Coupled Regulation of Evoked Dynorphin Release.** In the absence of hormone treatment, the  $K^+$ -evoked increment in dynorphin release was dose dependently inhibited by DPDPE ( $F_{4,21} = 18.373$ ,  $p < 0.05$ ; Fig. 4). Maximum



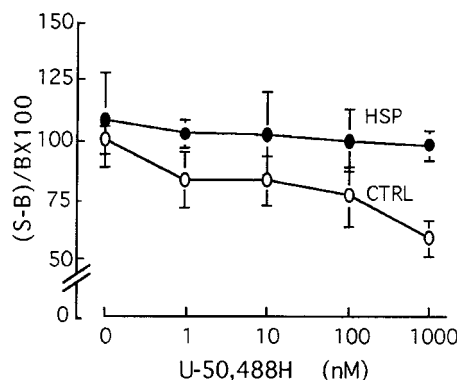
**Fig. 4.** Pregnancy levels of  $E_2/P$  fundamentally alter  $\delta$ -opioid receptor-coupled modulation of  $K^+$ -stimulated dynorphin release. Basal and  $K^+$ -stimulated release of dynorphin, in the absence or presence of the indicated concentration of DPDPE, was determined in spinal tissue obtained from control (○) or  $E_2/P$ -treated animals (●). Each point represents the mean  $\pm$  S.E.M. percent increase obtained from five to six independent experiments. In control spinal tissue, the  $\delta$ -opioid selective agonist DPDPE produced a monophasic dose-dependent inhibition of evoked dynorphin release. In spinal tissue obtained from  $E_2/P$ -treated animals, DPDPE continued to modulate the  $K^+$ -evoked release of dynorphin, but there was a biphasic (bell-shaped) reversal from inhibition to facilitation of stimulated dynorphin release. CTRL, control; S, stimulated; B, basal.

inhibition at 1  $\mu$ M was  $\sim 80\%$ . In spinal tissue obtained from  $E_2/P$ -treated animals, DPDPE continued to modulate the  $K^+$ -evoked release of dynorphin ( $F_{4,21} = 3.8$ ,  $p < 0.05$ ). However, in contrast to the monophasic inhibition observed in control tissue, the DPDPE concentration-effect curve was now bell-shaped, the initial region of which revealed an enhancement of  $K^+$ -stimulated dynorphin release (Fig. 4). This profound change in the DPDPE concentration-effect curve reveals striking plasticity in the transduction of signals generated by the  $\delta$ -opioid receptor and/or its spinal distribution.

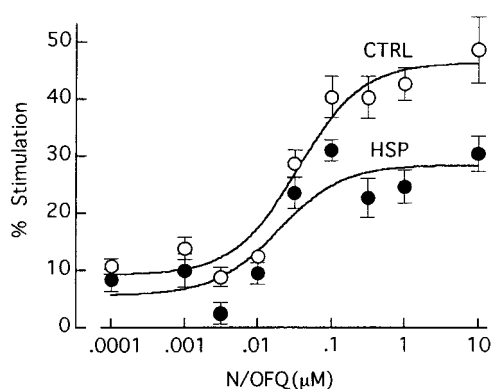
Simulation of the pregnancy blood profile of  $E_2$  and P also eliminated the ability of the  $\kappa$ -opioid receptor-selective agonist, U50,488H, to negatively modulate evoked dynorphin release (Fig. 5). In the absence of  $E_2/P$  treatment, U50,488H produced a dose-dependent (albeit modest) inhibition of evoked dynorphin release ( $F_{4,22} = 2.931$ ,  $p < 0.05$ ; maximum inhibition at 1  $\mu$ M =  $\sim 40\%$ ). In contrast, in spinal cord obtained from hormone-treated animals, the U50,488H concentration-effect relationship failed to reach statistical significance ( $F_{4,22} = 0.063$ ,  $p > 0.05$ ).

In contrast to spinal  $\delta$ - and  $\kappa$ -opioid receptors, activation of  $\mu$ -opioid receptors (via sufentanil, up to 1  $\mu$ M) failed to produce a significant concentration-dependent inhibition of stimulated dynorphin release ( $n = 5-6$  for each concentration; data not shown). Thus, spinal opioid receptor-coupled regulation of evoked dynorphin release is mediated predominantly via the  $\delta$ -type of opioid receptor. In this regard, it is interesting to note that the opioid analgesia associated with pregnancy and its hormonal simulation is mediated via spinal  $\delta$ - and  $\kappa$ - but not the  $\mu$ -opioid receptor (Dawson-Basoa and Gintzler, 1996, 1997a).

**Effect of the Pregnancy Profile of  $E_2/P$  on N/OFQ-Stimulated GTP $\gamma$ S Binding.** Consistent with previous observations (Narita et al., 1999), N/OFQ produced a concentration-dependent and saturable stimulation of [ $^{35}$ S]GTP $\gamma$ S binding (Fig. 6). In lumbar spinal cord membranes obtained from vehicle-treated animals, maximal stimulation was  $46.6 \pm 4\%$  with an  $ED_{50}$  of  $35.4 \pm 2.5$  nM. In contrast, in lumbar membranes obtained from  $E_2/P$ -treated animals, the maximal stimulation was  $29.1 \pm 3\%$ , with an  $ED_{50}$  of  $20.5 \pm 2$  nM ( $n = 6$  for both groups;  $p < 0.002$  and  $p < 0.005$  for maximal stimulation and  $ED_{50}$ , respectively, using paired



**Fig. 5.** Pregnancy levels of  $E_2/P$  abolish  $\kappa$ -opioid receptor-coupled inhibition of  $K^+$ -evoked dynorphin release. Basal and  $K^+$ -stimulated release of dynorphin, in the absence or presence of the indicated concentrations of the  $\kappa$ -opioid selective agonist U50,488H, was determined in spinal tissue obtained from control (○) or  $E_2/P$ -treated animals (●). Each point represents the mean  $\pm$  S.E.M. percent increase obtained from five to six independent experiments. CTRL, control; S, stimulated; B, basal.



**Fig. 6.** Pregnancy levels of  $E_2/P$  alter N/OFQ stimulation of GTP $\gamma$ S binding. Lumbar spinal cord membranes from one control and one hormone-treated animal were prepared and assayed in parallel as described under *Materials and Methods*. [ $^{35}$ S]GTP $\gamma$ S binding without added N/OFQ, in the absence or presence of 10  $\mu$ M unlabeled GTP $\gamma$ S, was determined in every assay and is defined as basal and nonspecific binding, respectively. The latter was subtracted from basal and stimulated values. Data, obtained from six independent pairs of membrane preparations and expressed as mean  $\pm$  S.E.M. of the percent stimulation over basal binding, were fitted to a sigmoidal dose-response curve using GraphPad Prism (San Diego, CA). Maximal stimulation produced by 10  $\mu$ M N/OFQ was abolished by the addition of the N/OFQ antagonist compound 15 (10  $\mu$ M). CTRL, control.

two-tailed *t* test). Basal activity did not vary among treatment groups ( $28.0 \pm 4.0$  and  $25.7 \pm 1.6$  fmol/mg of protein for tissue obtained from control and HSP animals, respectively). To validate the nature of the receptor mediating stimulated [ $^{35}$ S]GTP $\gamma$ S binding, the assay was conducted in the presence of the N/OFQ antagonist compound 15 (10  $\mu$ M). The presence of this antagonist abolished N/OFQ (10–10,000 nM) stimulation of GTP $\gamma$ S binding (data not shown).

## Discussion

The antinociception associated with pregnancy and its hormonal stimulation results from augmented activity of spinal  $\kappa$ - and  $\delta$ -opioid antinociceptive systems (Dawson-Basoa and Gintzler, 1996, 1998; Liu and Gintzler, 1999). The current results demonstrate that exposure of nonpregnant ovariectomized animals to pregnancy levels of  $E_2/P$  results in a significant increase in both the basal and stimulated rates of lumbar dynorphin release. This indicates that at least some of the regulatory processes that underlie ovarian sex steroid-induced opioid antinociception are presynaptic.

Contribution of presynaptic mechanisms to HSP antinociception is consistent with the increased content of dynorphin A (1–17) in lumbar spinal tissue obtained from pregnant (Medina et al., 1993a,b) or HSP animals (Medina et al., 1993a,b). It is also consonant with 1) the decreased content of dynorphin precursor intermediates (Medina et al., 1995) and 2) increased content of the prohormone processing enzyme prohormone convertase 2 in the lumbar spinal cord of pregnant and HSP animals (Varshney et al., 1999). Both suggest that increased processing of dynorphin precursor intermediates to mature dynorphin A (1–17) is a major adaptation by which spinal dynorphin/ $\kappa$ -systems adapt to increased demands of pregnancy.

In control lumbar tissue, the facilitative effect of the removal of spinal opioid tone (via naloxone) on  $K^+$ -evoked dynorphin release indicates that activation of endogenous spinal opioid systems can negatively modulate depolariza-

tion-induced dynorphin release. This interpretation is consistent with the demonstration that DPDPE and, to a lesser extent, U50,488H dose dependently inhibit the  $K^+$ -evoked release of spinal dynorphin. Similarly, the loss of this facilitative effect of naloxone on evoked dynorphin release following *in vivo* treatment with  $E_2/P$  indicates that pregnancy blood levels of ovarian sex steroids abolish this negative opioid modulation, a conclusion validated by the parallel loss of the ability of either DPDPE or U50,488H to inhibit the evoked release of dynorphin. In fact, in spinal tissue obtained from hormone-treated animals, a biphasic reversal from DPDPE-induced inhibition to enhancement of  $K^+$ -stimulated dynorphin release is observed. This reveals an ovarian steroid-induced conversion from negative to positive opioid receptor cross-talk among different opioid antinociceptive systems that could represent an antinociceptive feed-forward mechanism. The molecular underpinnings of this reversal are not known, but notably it is reminiscent of the chronic morphine-induced shift from opioid receptor-coupled inhibition to stimulation of met-enkephalin release and cAMP formation (for review, see Gintzler and Chakrabarti, 2000). These changes result from increased prominence of the stimulatory  $G_{\beta\gamma}$  signaling arm of G protein-coupled signaling (Chakrabarti et al., 2001).

Simulation of the pregnancy blood profile of  $E_2/P$  also elicits changes in the spinal cord N/OFQ system. The ability of N/OFQ to inhibit  $K^+$ -evoked dynorphin release in a concentration-dependent and naloxone-insensitive manner implies that endogenous N/OFQ represents an additional negative modulator of spinal dynorphin release. This is underscored by the demonstration that acute treatment with a selective N/OFQ antagonist significantly enhances the  $K^+$ -induced release of dynorphin from spinal tissue obtained from control animals. The abolishment of the exogenous N/OFQ inhibition of evoked dynorphin release from spinal tissue obtained from  $E_2/P$ -treated animals implies that, following the hormonal stimulation of pregnancy, spinal dynorphin neurons are no longer subject to negative modulation by endogenous N/OFQ. This contention is supported by the failure of the N/OFQ antagonist to augment the  $K^+$ -induced percent increment in dynorphin release from lumbar spinal tissue obtained from HSP animals, despite the maintenance of “normal” spinal levels of N/OFQ.

Interestingly, in some cases, the pharmacology of basal and evoked dynorphin release differed. For example, evoked but not basal release of dynorphin is negatively modulated by endogenous N/OFQ (it is enhanced by compound 15). Moreover, in HSP animals, evoked release of dynorphin is no longer negatively modulated by endogenous opioids (naloxone fails to enhance release), but such modulation of basal release persists. Such differences are not surprising, particularly in an *ex vivo* spinal cord preparation that, by necessity, is deafferented and thus lacks normal excitatory input. Voltage dependence of transmitter-gated activity is well documented. For example, cell depolarization in addition to glutamate is required for maximal activation of the *N*-methyl-D-aspartate-type channel. Furthermore, many ligand-gated effects result from modulation of a  $Ca^{2+}$ -dependent conductance that might not be present in the absence of depolarization.

[ $^{35}$ S]GTP $\gamma$ S binding is a commonly used indicator of receptor activation of G proteins. The statistically significant dec-

rement in the maximal N/OFQ stimulation of GTP $\gamma$ S binding in the lumbar cord of HSP animals reflects a reduction in functional NORs, presumably those associated with regulation of dynorphin release. On the other hand, the statistically significant decrease in ED<sub>50</sub> in hormone-treated spinal preparations could suggest the occurrence of a reciprocal increase in functionality of NORs present on other spinal neuronal phenotype(s), which now represent a larger fraction of the total receptor population than before hormone treatment. This observed increase in N/OFQ potency could result from a concomitant conformational/phosphorylation state change. The differential distribution of NORs among these spinal neuronal systems could account for the discrepancy between the magnitude of reduction in N/OFQ inhibition of dynorphin release (abolishment) and the magnitude of reduction in N/OFQ stimulation of GTP $\gamma$ S binding ( $\approx 37\%$ ). Moreover, given the association of NORs with the regulation of multiple spinal neuronal phenotypes, the observed E<sub>2</sub>/P-induced decrease in N/OFQ stimulation of GTP $\gamma$ S binding, a global indicator of N/OFQ functionality, is most likely an underestimate of hormone-induced plasticity.

In the spinal cord, specific blockade of NOR, but not opioid or other G protein-coupled receptors, alter N/OFQ-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding (Narita et al., 1999). This suggests that spinal N/OFQ pathways represent a distinct modulating entity. Moreover, the mRNA encoding NOR, as well as its associated protein, is present throughout the dorsal horn of the spinal cord, particularly in the superficial laminae (Harrison and Grandy, 2000). These regions process nociceptive stimuli and are enriched in dynorphin and enkephalin peptides. Thus, suggested interactions among spinal opioid and N/OFQ systems are consonant with their anatomy. The present data suggesting that the E<sub>2</sub>/P-induced increase in spinal dynorphin neuronal activity results, at least in part, from its disinhibition does not preclude contributions of postsynaptic plasticity (increased opioid receptor density, G protein coupling, etc.) to HSP-induced augmented spinal opioid tone.

Actions of N/OFQ in the central nervous system include hyperalgesia, reversal of opioid-mediated analgesia, allodynia, and even analgesia. Intracerebroventricular (i.c.v.), but not intrathecal (i.t.) N/OFQ attenuates the antinociception produced by exogenous opioids (Tian et al., 1997). In contrast, i.t. N/OFQ (Tian et al., 1997) and some of its shorter fragments (Rossi et al., 1997) have been reported to produce antinociception. Potentiation of systemic morphine antinociception by i.t. N/OFQ (Tian et al., 1997) has also been reported. On the other hand in rats, using hot plate, warm-water, or radiant heat tail-flick tests, N/OFQ administered either i.c.v. or i.t., failed to alter nociceptive responsiveness (Vanderah et al., 1998), but Hara et al. (1997) reported allodynia and hyperalgesia following i.t. N/OFQ. In the brain stem, N/OFQ inhibits two distinct groups of neurons to cause either a hyperalgesia, via the removal of  $\mu$ -opioid analgesia, or analgesia (Pan and Hirakawa, 2000).

One basis for complex physiology of N/OFQ is its ability to differentially alter multiple parameters of the same system. N/OFQ (i.t.) can enhance the release of spinal substance P (SP) and thereby promote nociception; but, at higher i.t. concentrations, it can also act postsynaptically to inhibit the actions of SP (Inoue et al., 1999). N/OFQ-induced enhanced release of SP could explain its ability to abolish the antino-

ception of physiological gestation and its hormonal stimulation (Dawson-Basoa and Gintzler, 1997b), despite its attenuated ability to inhibit stimulated spinal dynorphin release during HSP. The neurochemical basis for the abolition of gestational and HSP antinociception by i.t. N/OFQ awaits identification. The present results clarify, however, that the ability of N/OFQ to abolish HSP antinociception is not mediated via (direct) modulation of dynorphin release.

Spinal dynorphin has been associated with hyperalgesia, neurotoxicity, and analgesic tolerance to opioids (Dubner and Ruda, 1992; Vanderah et al., 2000). These actions represent the antithesis of the nontolerance-forming antinociceptive consequences attributed to the activation of spinal  $\kappa$ -opioid receptors during gestation and HSP (Dawson-Basoa and Gintzler, 1996, 1998; Liu and Gintzler, 1999). These disparate observations, however, are not necessarily incompatible since they assess spinal dynorphin/ $\kappa$ -functionality under vastly different states. The opposite role of spinal dynorphin in gestational and HSP antinociception suggests that activation of the spinal methionine-enkephalin/ $\delta$ -opioid system (Dawson-Basoa and Gintzler, 1997a; Liu and Gintzler, 1999) that occurs in combination with dynorphin/ $\kappa$ -pathways converts a hyperalgesic response to one of analgesia.

Concomitant increases in afferent activity could also be very influential in altering the balance among the varied effects ascribed to spinal dynorphin such that its antinociceptive actions are predominant. For example, concomitant activation of SP and opioid receptors not only enhances antinociceptive consequences of the latter, but produces opioid-dependent analgesia without the loss of potency (Foran et al., 2000). Collectively, these observations indicate that afferent input (via the hypogastric nerve), augmented during pregnancy and its hormonal stimulation, could be a critical determinant of the facet of dynorphin functionality that is manifest (see Gintzler et al., 1983; Liu and Gintzler, 1999).

In summary, in addition to demonstrating the importance of spinal  $\delta$ -opioid and N/OFQ receptor systems to the endogenous inhibition of dynorphin release, the current study reveals some of the mechanisms that underlie an opioid analgesia that is not accompanied by tolerance formation. Further elucidation of its components should provide a basis for the elaboration of pharmacotherapies, the usefulness of which is not restricted by the extreme loss of potency over time that has been the bane of the sustained use of narcotics.

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