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Estrogens synthesized and acting within a spinal oligomer suppress spinal endomorphin 2 antinociception: ebb and flow over the rat reproductive cycle

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1. Introduction

In humans and laboratory animals, nociception/opioid antinociception are sexually dimorphic and vary over the reproductive cycle [22; 30; 31; 41; 63; 76; 97]. We previously reported [63] that male and female rats differ in their responses to the highly selective endogenous μ -opioid receptor (MOR) ligand endomorphin 2 (EM2), the predominant endomorphin in the spinal cord [69; 104]. A striking feature of this sexual dimorphism is that the antinociceptive effectiveness of intrathecal (i.t.) EM2 is synchronized with the estrous cycle: the spinal EM2 antinociceptive system alternates between analgesically active and inactive states [63]. Interestingly, this characteristic is not manifested by other MOR ligands, e.g., sufentanil or [d-Ala²,N-methyl-Phe⁴,Gly-ol⁵]-enkephalin (DAMGO) [63], consistent with reports that EM2 stimulates specific MOR-coupled signaling pathways that differ from those activated by sufentanil or DAMGO [63; 73; 85]. Paradoxically, spinal EM2 analgesic responsiveness is robust (comparable to males) during proestrus and minimal during diestrus, notwithstanding that circulating levels of estrogens are low during diestrus (more comparable to males) and highest during proestrus [1; 14; 20; 38; 94; 96].

Notably, in female rats, 17- β -estradiol (estradiol), the predominant estrogen, can be pronociceptive [12; 43; 44; 60; 65; 88], as well as antinociceptive [4; 15; 32; 34; 50; 55; 66; 84; 89]. Modulation of nociception/opioid antinociception by estrogens occurs, in part, via classical nuclear estrogen receptors (ERs), which function as estrogen-activated transcription factors [21]. However, estrogenic modulation of nociception/opioid antinociception can also occur via ERs (α and β) that traffic to the plasma membrane (mER) [81] subsequent to being palmitoylated [59]. GPR30, a G protein-coupled ER [10; 16; 28; 95] also transduces membrane estrogen signaling [29; 83]. mERs localize to caveolae, where they activate

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multiple signaling cascades [74; 75; 99; 100]. In addition to differing in their subcellular localization (plasma membrane vs. nucleus) and their physiological consequences (activation of second messengers/signaling cascades vs. activation of transcription), mERs and nuclear ERs differ in their temporal profile of action (seconds/minutes vs. hours/days, respectively, for the onset and offset of effects).

Since estradiol can both positively and negatively modulate nociception/opioid antinociception, the apparent linkage of spinal EM2 analgesic responsiveness with stage of estrous cycle could result from either (1) enhanced facilitation by estrogen of spinal EM2/MOR antinociception (which, based on circulating levels of estrogens, would be expected to be greater in proestrus than diestrus), (2) a switch in estrogenic modulation from inhibitory in diestrus to facilitative during proestrus, or (3) loss during proestrus of the diestrus-associated negative estrogenic modulation. In order to investigate the nature of the physiological modulation of spinal EM2 antinociception by estrogens, we determined the effects of acutely manipulating spinal estrogenic tone during diestrus and proestrus on spinal EM2 analgesic responsiveness.

Paradoxically, our findings revealed a striking inverse relationship between estrogenic inhibition of spinal EM2 analgesic responsiveness and circulating levels of estrogens. This results from stage of estrous cycle-dependent interactions among components of a novel signaling oligomer. The oligomerization of aromatase with the plasma membrane-associated signaling receptors ER α , MOR and mGluR $_1$ provides a novel biochemical framework for estrogenic influences on nociception that can be regulated independent of ovarian production of estrogens.

2. Methods

2.1 Experimental animals

We used female rats (Sprague–Dawley; Charles River, Kingston, NY; 225–275 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 Committees of State University of New York Downstate Medical Center.

2.2 Determination of estrous cycle stage

Histology of vaginal smears was used to determine the stage of estrous cycle pre- as well as post-surgery. Predominance of small leukocytes was indicative of diestrus; predominance of large round nucleated cells indicated proestrus.

2.3 Implantation of intrathecal cannula

A permanent indwelling cannula was inserted into the lumbar spinal cord subarachnoid space under isoflurane anesthesia. In brief, a saline filled catheter (PE-10) was inserted through an incision in the atlanto-occipital membrane, slowly introduced into the spinal cord subarachnoid space (8.0 cm), and secured in place. The cephalic portion of the catheter was externalized through the skin above the skull area, where it was relatively inaccessible to the

paws. Only animals that appeared to be free of infection upon gross inspection were used. Motor integrity was assessed in all experimental groups by using the righting reflex and the inclined plane test. Those exhibiting any motor impairment following surgery were eliminated from the study. Experiments were initiated as early as one week after surgery and completed within two weeks thereafter. In order to eliminate any possibility that surgery confounded data interpretation by disrupting the estrous cycle, diestrus and proestrus were defined by vaginal smear histology, rather than predictions that assumed regularity of cycling.

2.4 Intrathecal administration of drugs and behavioral testing

1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydro-chloride (MPP, an ER α -selective antagonist), 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazol-ol[1,5-a]pyrimidin-3-yl] phenol (PHTPP, an ER β -selective antagonist), G-15 (a GPR30 antagonist), fadrozole (an aromatase inhibitor), 6-amino-*N*-cyclohexyl-*N*,3-dimethylthiazolo[3,2- α]benzimidazole-2-carboxamide hydrochloride (YM298198, an mGluR $_1$ antagonist) and 6-amino-*N*-cyclohexyl-*N*,3-dimethylthiazolo[3,2- α]benzimidazole-2-carboxamide hydrochloride (LY341495, an mGluR $_{2/3}$ antagonist) were each prepared in 3 μ l dimethyl sulfoxide; EM2 was prepared in 5 μ l 3% dimethyl sulfoxide. All drugs were obtained from Tocris (Ellisville, MO), except for fadrozole, which was obtained from Sigma (St Louis, MO). Drugs were applied to the spinal cord subarachnoid space over a 60 sec period via the indwelling i.t. cannula. Complete delivery was ensured by flushing the cannula with an additional 10 μ l of saline. I.t. doses of ER antagonists and fadrozole were based on our previous studies and chosen to maximize targeted effects while minimizing off target actions [62]. Doses of mGluR $_1$ and mGluR $_{2/3}$ antagonists were based on prior studies ([19; 33; 49; 98] and [48; 103; 108], respectively), as was the dose of EM2 [63; 102]. In order to demonstrate involvement of rapid signaling mER α , we administered i.t. EM2 as early as five min after i.t. MPP. In all subsequent experiments, in order to minimize possible confounds resulting from effects of accumulating volumes of i.t. injectate, we applied i.t. EM2 thirty min following blockade of ER α , ER β , GPR30, or mGluRs. In order to allow for run-down of spinal pools of estrogens, inhibition of spinal aromatase was started one hour prior the administration of EM2, as we performed in an earlier study [62]. Importantly, a number of studies have indicated the absence of significant supraspinal diffusion/effects of i.t. injectate even 60 min following i.t. injection [3; 46; 58].

Assessment of Tail Flick Latency—Nociceptive response thresholds were assessed using radiant heat as the nociceptive stimulus. Tail flick latency (TFL) was quantified using a Tail Flick Analgesia Meter (IITC, Woodland Hills, CA). Intensity of the radiant heat was adjusted such that baseline TFL were in the range of 3.0–4.5 sec. A cutoff of 10 sec latency prevented any untoward consequences to the tail. Data are expressed as either absolute TFL or percent change.

2.5 Membrane preparation, immunoprecipitation (IP) and Western blot analysis

Spinal cord membranes were prepared and solubilized as described previously by this laboratory [17]. Briefly, animals were sacrificed by decapitation; spinal tissue was homogenized in cold 20 mM HEPES, pH 7.4, containing 10% sucrose, 5 mM EDTA, 1 mM

EGTA, 2 mM dithiothreitol, and multiple protease inhibitors [1 mM benzamidine, 0.2 g/L Bacitracin, 2 mg/L Aprotinin, 3.2 mg/L each of Leupeptin and trypsin inhibitor from soybean; 20 mg/L each of N-tosyl-L-phenylalanine chloromethyl ketone, Na-*p*-tosyl-L-lysinechloromethyl ketone and phenylmethylsulfonyl fluoride, and cOmplete™ cocktail protease inhibitor tablet/50 ml (Roche Molecular Biochemicals, Indianapolis, IN); for experiments assessing the phosphorylated state of ER α , PhosSTOP™ cocktail phosphatase inhibitor tablet/10ml (Roche) was also added]. Supernatants from a low-speed spin (1,000 g for 10 min, 4°C) were centrifuged at a higher speed (30,000 g for 40 min, 4°C) to obtain (crude) membrane pellets. Membranes were solubilized in the above buffer without sucrose and dithiothreitol but now containing 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% Na-deoxycholate, 0.1% Na-dodecyl sulfate and 10% glycerol, agitated for 60 min at 4°C and centrifuged (16,000 g for 40 min at 4°C). IPs were obtained from the clear supernatant by overnight incubation (4°C) of equivalent membrane protein (measured by Bradford assay) with specific antibodies and Protein A or G agarose beads. IP samples were washed, eluted with heat in SDS sample buffer, separated on SDS-PAGE, transferred onto nitrocellulose membrane, and Western blotted.

Co-immunoprecipitates (co-IPs) to be compared were always obtained and processed in parallel. Western quantification of the co-immunoprecipitated (co-IPed) protein was always normalized against the protein targeted for direct IP, which was also quantified by Western analysis using antibodies against a different epitope. Moreover, Westerns of all IPed proteins (those directly targeted as well as co-IPed) utilized antibodies raised in a host different from that used for the generation of antibodies used for IP to avoid cross-recognition by secondary antibodies. Antibody–substrate complex was visualized using Supersignal West Dura kit (Pierce). Chemiluminescence was captured via G:Box CCD camera (Syngene, Frederick, MD) and intensities quantified using Genetools software (Syngene).

Specificity of all Western signals (that of the directly IPed as well as co-IPed proteins) was validated by their absence when Western blotting using preadsorbed antibody: antigenic peptide used for generation of the antibody was diluted in 20 mM HEPES, pH 7.4, at five times excess to antibody and covalently coupled to Affi-Gel® 10 Gel matrix in accordance with the manufacturer's instructions (Bio-Rad). The desired antibody was added in TBS and allowed to preadsorb. Following incubation, the flow-through was collected ("preadsorbed antibody") and used to probe one of two identical nitrocellulose membrane strips containing the protein of interest (the other strip was probed in parallel with an equivalent amount of primary antibody and the difference in signal intensity quantified). The specificity of the phosphoSer118ER α (pSER α) Western signal was validated by the ability of the anti-pSER α -antibody to recognize the eleven aa phosphoSer118 antigenic peptide, but not its nonphosphorylated congener.

Blue native PAGE/SDS-PAGE and sequential immunoprecipitation—Spinal membranes were solubilized in 20 mM HEPES buffer, pH 7.4, containing 1 mM EDTA, protease inhibitors as above, and 2% mild detergent digitonin, incubated on ice (60 min) and centrifuged (15,000 g, 20 min, 4 °C). Native sample buffer and the anionic dye Coomassie blue G-250 were added to spinal membrane supernatant and electrophoresed using 3–12% Blue Native gels (Invitrogen). Blue native PAGE was carried out as previously described

[90]. The migration distances of individual bands relative to marker proteins were used to estimate native masses of membrane proteins. A ≈ 360 kDa band was initially identified with Western analysis using either anti-MOR or anti-ER α antibodies. In subsequent experiments, a band of comparable molecular mass was stained with Coomassie blue and excised. Native proteins were eluted with 50 mM Tris-HCl containing 150 mM NaCl, and 0.1 mM EDTA; pH 7.5. Eluted proteins were separated using SDS-PAGE and immunoblotted for aromatase, ER α , MOR and mGluR $_1$.

For sequential IP, supernatants containing known protein concentrations (Bradford) were subjected to IP utilizing (in order) antibodies against aromatase, ER α , and mGluR $_{1a}$. Samples were incubated with antibody cross-linked to Protein A or G agarose beads using DSS (Pierce), washed and gently eluted with 100 mM glycine, pH 2.5. Eluate was neutralized (1 M Tris, pH 8.8) and incubated with the next antibody. The final IP was eluted with 100 mM glycine. Native sample buffer and Coomassie blue were added to eluted final IP, which was followed by electrophoresis using 3–12% Blue Native gels and electro-transfer onto PVDF membranes. Anti-MOR Western analysis was performed as reported [17]. Since we had previously demonstrated the specificity of MOR Western signals in spinal cord [17; 64], this control was not repeated in the present study.

2.6 Immunocytochemistry (ICC)

Diestrous female rats were deeply anesthetized with a mixture of ketamine (68 mg/kg), xylazine (4.6 mg/kg), and acepromazine (0.9 mg/kg) and perfused through the ascending aorta with 100 ml of ice-cold oxygenated calcium-free Tyrode's buffer (115 mM NaCl, 5 mM KCl, 2 mM MgCl $_2$ ·6H $_2$ O, 400 μ M MgSO $_4$ ·H $_2$ O, 3 mM glucose, and 25 mM NaHCO $_3$, pH 7.2) followed by 500 ml of freshly prepared fixative (4% w/v paraformaldehyde, 14% v/v saturated aqueous picric acid, 75 mM KH $_2$ PO $_4$, and 85 mM Na $_2$ HPO $_4$ ·7H $_2$ O, 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 $_4$, and 70 mM Na $_2$ HPO $_4$ ·H $_2$ O, pH 7.2), and shipped via overnight air courier service to Minnesota on refrigerant gel packs.

Upon arrival, tissue was quickly frozen and serial sections were cut to a nominal thickness of 5 μ m on a cryostat (Bright Instruments, Huntington, UK), thawed on Probe-On Plus microscope slides (Fisher Scientific, Pittsburgh, PA) and stored at -20° C until used. Sections were rinsed in distilled water and immersed in 10 mM trisodium citrate, pH 6.0, containing 0.05% Tween-20 and autoclaved at 101° C for 30 minutes. The sections were allowed to cool to room temperature, rinsed in TBS (135 mM NaCl, 4 mM KCl and 25 mM Tris-HCl, pH 7.4), then incubated in permeabilization solution (TBS containing 0.2 % Triton X-100 and 0.2 % Tween-20) for one hour at room temperature. Sections were incubated with guinea pig anti-MOR $_1$, mouse anti-mGluR $_1$, and either rabbit anti-ER α , rabbit anti-EM2 or rabbit anti-aromatase. All staining with the MOR antibody was abolished when used on MOR knockout mice (not shown). For anti-ER α , the staining specificity was previously validated by confirming immunohistochemical results with a second anti-ER α antibody generated against a different epitope [92] and by preadsorption [107]. Staining specificity of our anti-EM2 antibody in rat has been previously demonstrated [80]. Notably, although EM2 has an amidated C-terminal phenylalanine, which is found in other peptides such as NPFF, this is

not sufficient for their detection in tissue by the anti-EM2 antibody employed in this study [80]. Moreover, it is highly unlikely that EM2 staining results from the antibody recognizing either substance P or CGRP, given that the antibody does not detect EM2-ir in the lateral spinal nucleus [80], where SP is expressed [36], or in ventral horn motor neurons, where CGRP is expressed [35].

Sections were washed and then incubated in a mixture of 3 µg/ml each of donkey anti-rabbit IgG conjugated to Cy3, donkey anti-guinea pig IgG conjugated to Cy2 and donkey anti-mouse IgG conjugated to Cy5 (Jackson ImmunoResearch). The sections were washed, dehydrated, cleared in xylene, and mounted with cover slips using DPX (Fluka, Ronkonkoma, NY).

Microscopy—Images were collected using an Olympus Fluoview 1000 confocal microscope equipped for excitation at 488 nm (Cy2), 543 nm (Cy3) and 635 nm (Cy5). Images were collected with 40× 1.3 NA or 60× 1.4 NA objectives and pixel dimensions of 0.32 µm or smaller; all images were made using sequential scanning to avoid bleed-through. Photoshop was used to pseudocolor images and to adjust brightness and contrast.

2.7 Antibodies

The following antibodies were used for (1) **IP**: anti-mGluR₁ (sc-47131; 2 µg per 800 µg of total protein), anti-ERα (sc-71064; 3 µg per 800 µg of total protein), anti-aromatase (sc-14245; 3 µg per 800 µg of total protein), all purchased from Santa Cruz Biotechnology (SCB); (2) **Western blot**: anti-mGluR₁ (US Biologicals, M3884-75C; 1:1000), anti-ERα (SCB, sc-542; 1:250), anti-aromatase (abcam, ab124776; 1:40,000), anti-GPR30 (SCB, sc-48524-R; 1:250), anti-MOR (raised in rabbit against carboxyl-terminal 50 aa of MOR; generous gift from Dr. Thomas Cote; 1:1000) [18], anti-pSERα (SCB, sc-12915-R; 1:250); and (3) **ICC**: anti-ERα (SCB, sc-542; 1:1000) [62], anti-MOR₁ (raised in guinea pig against aa 384–398 of MOR₁; 1:1000) [5], anti-mGluR₁ (BD Biosciences, 610964; 1:1000), anti-aromatase (abcam, ab69653; 1:1000) [13], and anti-EM2 (generated by the Wessendorf laboratory against the full sequence of EM2 and affinity isolated; 1:1000) [79; 80].

2.8 Data analysis and statistics

One-way ANOVA was used to compare basal TFL among groups as well as to compare treatment effect within a group; two-way ANOVA was used to compare either treatment-by-time effects (within the same cycle stage) or cycle stage-by-treatment effects (at 5 min after i.t. EM2) on TFL. Unpaired Student's T-tests were used to analyze differences in Western signals of co-IPed proteins during diestrus vs. proestrus. Statistical comparisons were made using Prism 5 software.

3. Results

3.1 Spinal mER-coupled regulation of i.t. EM2 antinociception depends on stage of estrous cycle

Consistent with our earlier report [63], basal TFL did not significantly vary among groups. Additionally, we replicated our earlier finding that i.t. EM2 produces a significant dose-

dependent antinociception during proestrus, whereas during diestrus minimal or no antinociception was observed [63]. Since peak antinociception was observed at 45 nmol i.t. EM2 [63], this dose was used in the current study.

Current data analyses revealed a significant difference in treatment effect (blockade of individual mERs) on spinal EM2 (45 nmol) antinociception during diestrus, but not proestrus (Fig. 2A vs. Fig. 1). During proestrus, neither blockade of spinal ER α (via i.t. MPP, 10 nmol, n=6), ER β (via i.t. PHTPP, 10 nmol, n=4), nor GPR30 (via i.t. G-15, 10 nmol, n=4) altered spinal analgesic responsiveness to EM2; two-way ANOVA revealed a significant within group analgesic effect of EM2 ($F_{4,68}=17.03$, $p<0.0001$) but no effect of treatment (ER blockade; $F_{3,68}=0.79$, $p=0.51$). In contrast, during diestrus the same i.t. treatment with MPP or G-15, but not their vehicle, unveiled a robust i.t. EM2 antinociception (for i.t. MPP: $F_{4,16}=4.275$, $p=0.015$, n=5; Dunnett's Multiple Comparison Test: $p<0.05$ for basal TFL vs. TFL 5 min after i.t. EM2; for G-15: $F_{4,16}=4.301$, $p=0.015$, n=5; Dunnett's Multiple Comparison Test: $p<0.01$ for basal TFL vs. TFL 5 min after i.t. EM2; Fig. 2A). Importantly, the effect of MPP was manifest as early as 5 min after its spinal pretreatment ($F_{1,18}=6.21$, $p<0.05$ for the comparison between MPP+EM2 group vs. DMSO +EM2 group, n=4; Fig. 2A inset), suggesting the relevance of rapid mER signaling to spinal EM2 antinociception. Pretreatment with MPP or G-15 in the absence of EM2 (but in the presence of its vehicle) did not alter TFL (MPP: $F_{4,12}=0.047$, $p=0.75$; G-15: $F_{4,12}=1.944$, $p=0.17$; n=4 for both drugs; Fig. 2A). Moreover, the spinal EM2 antinociception (5 min after i.t. EM2) in MPP or G-15 pretreated diestrous rats was indistinguishable not only between these two groups ($F_{1,32}=0.009$, $p=0.926$), but also from the vehicle-pretreated proestrous group ($F_{2,28}=0.092$, $p=0.913$; Figs. 2A vs. 1). These results indicate that the transition from being unresponsive to i.t. EM2 to being analgesically responsive to it did not result from a qualitative shift in estrogenic modulation, but instead involved the *loss of its negative modulation*.

During proestrus, 15 nmol EM2 failed to elicit significant antinociception ($F_{4,16}=2.71$, $p=0.068$) whereas antinociception was elicited by both 45 ($F_{4,28}=5.64$, $p=0.002$) and 90 nmol ($F_{4,16}=4.47$, $p=0.013$). These responses were indistinguishable ($F_{1,44}=1.00$, $p=0.339$), suggesting that 45 nmol produced maximum antinociception. Notably, in diestrus, antinociceptive responses to i.t. EM2 following i.t. MPP were comparable to those observed in untreated proestrous rats. In diestrous rats pretreated with i.t. MPP, 15 nmol EM2 continued to be below threshold ($F_{4,12}=0.91$, $p=0.447$) and 45 nmol produced an antinociception ($F_{4,12}=10.61$, $p<0.001$) the magnitude of which did not differ from that observed in proestrous rats ($F_{1,40}=0.082$, $p=0.781$) (Fig. 2B).

In contrast to ER α or GPR30 blockade, blockade of ER β (via i.t. PHTPP) failed to enable spinal EM2 analgesic responsiveness during diestrus ($F_{4,20}=0.82$, $p=0.53$, n=6; Fig. 2A). The inability of i.t. PHTPP to unmask spinal EM2 antinociception did not result from inadequate blockade of spinal ER β since the same dose of i.t. PHTPP eliminates the ER β dependent heterodimerization of spinal MOR with kappa-opioid receptors (KOR) [62].

3.2 Locally synthesized estrogens regulate spinal EM2 analgesia

To determine if spinal ER-mediated suppression of i.t. EM2 antinociception requires locally synthesized estrogens, we investigated the effect of the intrathecally applied aromatase inhibitor fadrozole (2.5 nmol) (Fig. 2C). In diestrus, i.t. application of fadrozole (one hour pretreatment) unveiled a robust spinal EM2 analgesia ($F_{5,20}=7.221$, $p=0.0005$, $n=5$; Dunnett's Multiple Comparison Test: $p<0.01$ for basal TFL vs. TFL at 5 min following i.t. EM2), comparable in magnitude to the EM2 antinociception observed in proestrous rats (shown in Fig. 1) ($F_{1,20}=0.481$, $p=0.496$). Importantly, the same i.t. fadrozole pretreatment in the absence of i.t. EM2 (but in the presence of its vehicle) did not alter TFL ($F_{4,12}=2.45$, $p=0.103$, $n=4$; Fig. 2C). Since blockade of spinal ERs did not alter i.t. EM2 antinociception in proestrous rats (Fig. 1), the effect of i.t. fadrozole on spinal EM2 antinociception was not determined at this estrous stage.

3.3 Association of spinal aromatase with mER α and GPR30

The specific stage of the estrous cycle when spinal aromatase/mER α activity is physiologically relevant varies for different endpoints. For example, spinal aromatase/mER α activity is essential for the heterodimerization of MOR with KOR during proestrus [62], but during this cycle stage, spinal aromatase/mER α activity does not modulate spinal EM2 antinociception (although it does during diestrus). These differences imply that discrete populations of spinal mER α are independently activated by discrete pools of locally synthesized estrogens. The most parsimonious model to explain segregated activation of spinal mER α is one that posits its presence in spatially and functionally segregated plasma membrane-associated complexes with aromatase.

To test this hypothesis, we determined if spinal aromatase and mER α co-IP. The magnitude of aromatase IPed from spinal cords of diestrus vs. proestrous rats did not differ. Two previously reported molecular mass species of ER α (≈ 35 and ≈ 50 kDa) [9; 57; 105] were present in aromatase IP obtained from spinal membranes of both diestrus and proestrous rats (Fig. 3A, left panel, data shown only for diestrus). The magnitude of the co-IP of mER α with aromatase was not dependent on stage of estrous cycle, i.e., the signal intensity of neither the ≈ 35 nor the ≈ 50 kDa ER α species differed between diestrus vs. proestrous rats ($n=7$; unpaired t-test: $p>0.05$ for both comparisons, data not shown). These results indicate that aromatase and mER α are physically associated, and their association does not vary over the estrous cycle.

In order to ascertain if the association of aromatase with spinal mER α generalizes to GPR30, we also explored the presence of GPR30 in spinal aromatase IP. When aromatase IP was Western blotted with anti-GPR30 antibody, a signal was observed at ≈ 50 kDa (Fig. 3A, right panel; data shown only for diestrus), the reported molecular mass for GPR30 [109]. We did not explore the co-IP of aromatase with mER β since activity of this ER did not suppress i.t. EM2 antinociception during diestrus.

3.4 Aromatase-mER α complexes are functional units, varying in activity during diestrus vs. proestrus

ER α is phosphorylated in response to estrogens, predominantly on Ser118, which increases its activity [45; 54]. The rapidity (<5 min) of phosphoSer118ER α (pSER α) formation implies that it is a direct response to estrogen binding [45]. Thus, the content of pSER α in aromatase IP reflects the activation of the co-IPed mER α . This, in turn, indicates elevated production of local estrogens and thus the increased activity of mER α -associated aromatase. Accordingly, we quantified by Western analysis the co-IP of membrane pSER α with aromatase. Two molecular mass species of pSER α ($\approx$ 35 and $\approx$ 50 kDa) were observed (Fig. 4A). The mean signal intensity of both pSER α Western signals was significantly elevated in diestrous vs. proestrous rats ($p < 0.05$, $n = 6$; 32% and 50% for upper and lower bands, respectively; Fig. 4B). These results indicate that the activity of the mER α that is complexed with spinal aromatase (and presumably the activity of that aromatase), is elevated during diestrus, i.e., is inversely related to circulating levels of estrogens.

3.5 mGluR $_1$ is required to attenuate spinal EM2 analgesia in diestrus

mER α can act via mGluR $_1$ to activate cAMP response element binding protein (CREB) and to mobilize calcium [11; 24; 52]. Thus, we investigated if spinal mGluR $_1$ activity is required for the observed mER α -mediated suppression of i.t. EM2 analgesia during diestrus. As illustrated in Fig. 5, i.t. application of the non-competitive mGluR $_1$ antagonist YM298198 (25 nmol, 30 min; threshold $\approx$ 5 nmol) during diestrus rapidly unveiled a robust spinal analgesic response to i.t. EM2 ($F_{4,24} = 13.98$, $p < 0.0001$, $n = 7$; Dunnett's Multiple Comparison Test: $p < 0.01$ for basal TFL vs. TFL obtained 5 min after i.t. treatment with EM2), the magnitude of which was significantly greater than the minimal i.t. EM2 analgesia elicited in the absence of i.t. YM298198 ($F_{1,39} = 5.872$, $p = 0.031$; Bonferroni post-hoc tests: $p < 0.001$ at 5 min following i.t. EM2), but comparable to that observed following acute spinal mER α /GPR30 blockade ($F_{2,56} = 0.037$, $p = 0.96$). In contrast, mGluR $_{2/3}$ blockade (via 25 nmol LY341495) failed to unmask analgesic responsiveness to i.t. EM2 ($F_{4,24} = 1.176$, $p = 0.346$, $n = 7$; Fig. 5).

3.6 Association of mER α and mGluR $_1$ is augmented in spinal cord of diestrous vs. proestrous rats

ERs interact with and activate membrane receptors (e.g., epidermal growth factor receptor and insulin-like growth factor-1 receptor [47; 82]). We hypothesized that mER α activates mGluR $_1$ via analogous protein-protein interactions. The ability of mGluR $_1$ to be activated by mER α would thus be reflected by their co-IP. Accordingly, to determine whether spinal mER α -mGluR $_1$ signaling varied between diestrus and proestrus, we compared the presence of mER α in mGluR $_1$ IP obtained from spinal membranes of diestrous and proestrous rats. Two molecular mass species of mER α , comparable to those present in aromatase IP ($\approx$ 50 and $\approx$ 35 kDa), were present in mGluR $_1$ IP (Fig. 6A). Interestingly, the content of both species of mER α was significantly elevated in diestrous vs. proestrous rats ($\approx$ 50 kDa: 42%, $p = 0.027$; $\approx$ 35 kDa: 54%, $p = 0.008$; $n = 6$; Fig. 6B). This is consistent with mER α -mGluR $_1$ negative modulation of spinal EM2 analgesic responsiveness during diestrus, but not proestrus.

3.7 Presence of an oligomer containing MOR, mER α , mGluR $_1$, and aromatase in spinal cord

The co-IP of aromatase with mER α , and mER α with mGluR $_1$, as well as the ability of mER α -mGluR $_1$ signaling to negatively modulate i.t. EM2 antinociception, suggests the close physical association of aromatase, mER α , mGluR $_1$ and MOR. To investigate this possibility, spinal membranes were electrophoresed using Blue native gels. The ≈ 360 kDa band that was initially immuno-stained with either anti-MOR or anti-ER α antibodies and in subsequent experiments identified using Coomassie blue, was excised and eluted. Equal aliquots of the eluted band were subjected to reducing SDS-PAGE. Western analyses employing antibody against aromatase, ER α , mGluR $_1$ or MOR revealed that all were contained in the ≈ 360 kDa band that was visualized using native gel electrophoresis (Fig. 7A and B).

The apparent molecular mass of the ER α present in the ≈ 360 kDa oligomer (≈ 60 kDa) differed from the low molecular mass isoforms that associate with either mGluR $_1$ or aromatase, (≈ 50 kDa and ≈ 35 kDa, the latter being the one that was predominantly phosphorylated during diestrus). Isoform-specific associations observed for mER α are paralleled by variation in apparent molecular mass of MORs; whereas the ≈ 50 kDa species of MOR is present in the oligomer (Fig. 7), the ≈ 60 kDa MOR species predominantly heterodimerizes with kappa-opioid receptors [17]. Notably, the ≈ 50 kDa MOR species is a minor fraction of MORs present in spinal cord, the predominant molecular mass species being ≈ 60 and 75 kDa (data not shown and [56; 106]). This suggests that variation in the degree of N-linked terminal glycosylation of MOR (the most likely explanation for varying molecular mass) could influence its chemical partnering. Taken together, these observations indicate that biochemical context can be an important determinant of the relative participation/subcellular sorting of the different molecular mass species of a particular protein, and thus functional differences among them.

To validate that aromatase, mER α , mGluR $_1$ and MOR were indeed present in a common oligomer and not different complexes that co-migrated on native gels, we performed sequential IP using (in order) antibody against aromatase, ER α , and mGluR $_1$. The final IP was eluted, electrophoresed using Blue native gels and Western blotted with anti-MOR antibody, which revealed a ≈ 360 kDa band. This cross-validated the presence of a ≈ 360 kDa oligomer in spinal cord containing aromatase, mER α , mGluR $_1$ and MOR (Fig. 7C). Importantly, the presence of aromatase in a complex containing MOR and mGluR $_1$, which overwhelmingly localize to the plasma membrane, underscores the similar location of aromatase (and thus, the likely production of estrogens) despite the possible presence of microsomes in our crude membrane preparations.

3.8 Coexpression of MOR, mGluR $_1$, ER α , and aromatase in spinal neurons apposed by EM2 varicosities

If endogenous EM2 acts at least in part via cells expressing an oligomer of aromatase, mER α , mGluR $_1$ and MOR, there should exist at least some spinal neurons that coexpress these proteins and are apposed by EM2 varicosities. Although aromatase, mER α , mGluR $_1$ and MOR are distributed broadly in the dorsal horn, Fig. 8A (top panels) shows that these

signaling molecules are sometimes coexpressed in or near the plasma membrane of neurons in the superficial dorsal horn during diestrus. EM2 is also widely distributed in the dorsal horn, frequently not apposed to MOR, as previously reported [67; 68; 70; 79; 80]. This is consistent with reports that EM2 interacts with MOR via non-synaptic mechanisms [2; 93]. Nevertheless, Fig. 8B (bottom panels) shows an EM2-immunoreactive varicosity apposing the dendrite of a MOR-immunoreactive neuron in L6 spinal cord that coexpresses mGluR₁ and mER α . ER α is also abundantly expressed in the cell body as well as in or near the plasma membrane of spinal neurons. These ICC data provide a cellular basis for our proposal that spinal mER α , mGluR₁ interact within a neuron also producing estrogens to negatively modulate i.t. EM2 analgesia during diestrus (see Fig. 9 for schematic representation of our hypothesis).

ICC images show that MOR, aromatase, mGluR₁ and mER α largely exist in different parts of the cell. This appears to be at odds with our data pointing to their substantial membrane oligomeric organization, as shown by IP. However, it is important to note that IP and ICC are not quantitatively comparable since (1) the preparation used for IP contains a predominance of plasma membranes vs. the other intracellular compartments visible in the ICC images and (2) immunogenic epitopes within an oligomer may not be as accessible in fixed tissue as they are in the unfixed, solubilized membrane preparation. These considerations could also explain the profound effects of blocking spinal mER α or mGluR₁ on i.t. EM2 antinociception even though many of the MORs appear not to be juxtaposed with mER α or mGluR₁.

4. Discussion

This study demonstrates: (1) during diestrus, but not proestrus, spinal antinociceptive responsiveness to i.t. EM2 is actively suppressed by spinal mER α -mGluR₁ signaling; (2) spinally synthesized estrogens are required for this suppression; (3) aromatase, classically considered to be an intrinsic endoplasmic reticulum protein, is present in a spinal plasma membrane oligomer containing mER α , mGluR₁ and MOR; and (4) MOR-ir neurons in the superficial dorsal horn receiving EM2 input coexpress aromatase, mER α , and mGluR₁, suggesting the relevance of the above oligomer to endogenous EM2 antinociception.

Estrogenic suppression of i.t. EM2 antinociception likely results from rapid-signaling mERs since the effect of ER α blockade was manifest as early as 5 min following the i.t. pretreatment with MPP, shorter than the approximately 20 min required for transcription of even immediate early genes [53]. Moreover, physiological effects of proteins encoded by these genes would require translation, subsequent chemical modification and intracellular trafficking in addition to transcription, which would exceed both the 5 and 30 min pretreatment times employed. Thus, rapid signaling mER α /GPR30 are the most likely ERs that suppress spinal EM2 analgesic responsiveness.

Inhibition of spinal aromatase during diestrus (via i.t. fadrozole) unmasks i.t. EM2 antinociception, indicating that suppression of spinal EM2 antinociception during diestrus results from spinal ER activation by locally synthesized estrogens. This is consistent with the presence of aromatase in many spinal areas involved in nociception/opioid

antinociception [26; 27; 40; 77; 78]. Spinal ERs can also be activated by peripheral estrogens, which penetrate the blood-brain barrier [37]. The contribution of peripheral estrogens to suppression of i.t. EM2 antinociception was not explored in the current study since it is exceedingly problematic to selectively eliminate circulating estrogens without also eliminating other (ovarian) factors: an ovariectomized rat is not an appropriate control for an intact diestrous rat, notwithstanding their comparably low circulating levels of estrogens.

Estrogen activation of mER α is reflected by its phosphorylation [45; 54]. Thus, the significant (albeit modest) increase in membrane pSER α that co-IPed with aromatase from spinal cord of diestrous vs. proestrous rats, likely reflects an increase in the activity of the aromatase associated with mER α . Importantly, the increase in pSER α we observed is probably an underestimate, since the antibody used to IP aromatase is not selective for its activated form: both non-activated and activated mER α were co-IPed.

The inverse relationship between the activity of at least some populations of spinal aromatase and circulating concentrations of estrogens indicates that cycle stage does not reliably predict the magnitude of spinal estrogenic signaling. At present, mechanisms responsible for this out-of-phase relationship between spinal aromatase activity and circulating levels of estrogens are not known. However, this inverse relationship could underlie inconsistent findings regarding the magnitude of nociception/opioid antinociception over the estrous and menstrual cycle [23]. Additionally, the differential dependence of the activity of discrete pools of aromatase on stage of cycle could account for variable nociception/opioid antinociception across the reproductive cycle [22; 30; 31; 41; 63; 76; 97], and provides an imperative for including more than a single reproductive cycle stage when studying pain and its amelioration.

In cultured hippocampal pyramidal neurons, estradiol (via mER α) activates mGluR $_1$ (inducing CREB phosphorylation) [11]. Additionally, direct interactions between mER α and mGluR $_1$ activate MOR in the medial preoptic nucleus [24]. In the current study, it is possible that the i.t. EM2 antinociception unveiled during diestrus by mGluR $_1$ blockade resulted from the loss of mGluR $_1$ -mediated hyperalgesia, independent of any association of mGluR $_1$ with mERs and/or MOR. However, this is unlikely since i.t. application of the mGluR $_1$ blocker YM298198 alone had no effect on TFL.

The suggested functional association between spinal mER α and mGluR $_1$ is underscored not only by their co-IP, but also by the fact that the loss of mER α -coupled negative modulation of spinal EM2 antinociception during proestrus (reflected by the inability of spinal ER α blockade to increase spinal EM2 antinociception) is accompanied by the diminution of the mER α -mGluR $_1$ physical association (reflected by their reduced co-IP). We hypothesize that mER α activation is a prerequisite for its interaction with mGluR $_1$. In diestrus, increased spinal aromatase activity (resulting in increased estrogen production) and consequent mER α phosphorylation promote mER α 's interaction with mGluR $_1$. Conversely, in proestrus, reduced spinal aromatase activity, and thus reduced mER α phosphorylation, promote mER α -mGluR $_1$ disassociation. Although not tested in this study, it is likely that mGluR $_1$ also associates with GPR30, given that GPR30 associates with both aromatase (present findings) and mER α [51].

The robust spinal antinociceptive responsiveness to EM2 during proestrus could result from either the loss of mER α -/GPR30-coupled negative modulation or a shift in mER α /GPR30 modulation from inhibition to facilitation (e.g., E2 activation of mER α can both increase and decrease CREB activity in hippocampal neurons [11]). However, it is unlikely that facilitatory mER α signaling contributes to the emergence of EM2 antinociception during proestrus since blockade of spinal mER α did not diminish i.t. EM2 antinociception at this cycle stage. Thus, augmented EM2 analgesic responsiveness in proestrus most likely emerges from the loss of diestrus-associated mER α -/GPR30-coupled suppression.

Three main strategies have been described for estrogen's actions: endocrine, paracrine, and autocrine. These differ only with respect to the distance between the point of synthesis and the site of action (reviewed in [87]). Recently, a fourth, synaptocrine, has been proposed in the CNS [87], which involves specific modulation of synaptic estrogen concentrations. Synaptocrine estrogenic signaling is made possible by the presence of aromatase and ERs on dendritic/somatic membranes and presynaptic terminals [6–8; 71; 72; 91; 101]. The current demonstration of aromatase in membrane preparations, however, does not permit quantitative comparisons with previous studies demonstrating abundant aromatase in the cell body [25; 42], given differences in preparation and visualization techniques employed: IP concentrates aromatase and Western blots amplify the corresponding chemiluminescent signal.

Current findings provide an additional dimension of CNS estrogenic signaling. The oligomerization of aromatase and mERs with other plasma membrane-associated signaling molecules confers exquisite spatial and temporal specificity to estrogenic signaling. Furthermore, the presence of aromatase activity within a signaling complex containing mERs permits the activation of discrete subcellular ER-coupled signaling (e.g., via mGluR $_1$). We coin the term 'oligocrine' to describe the ability of estrogens to function as intra-cellular messengers that are synthesized and act within the same macromolecular signaling complex. The oligomeric segregation of aromatase-mERs allows the on/off switching of spinal mER signaling to be independent of estrous cycle-associated variations in circulating levels of estrogens – either out- of-phase (as in the current study) or in-phase with them (e.g., spinal mER-mediated facilitation of MOR/KOR heterodimerization [62]).

CNS oligocrine estrogenic signaling would complement endocrine (peripheral estrogens acting on CNS ERs) and neurotransmitter-like synaptocrine estrogenic signaling. The presence of multiple modalities of CNS estrogenic signaling suggest the importance of coordinating not only the actions of peripheral and central estrogens, but also the activity of spatially and, presumably, functionally segregated pools of aromatase in the CNS. Coordination of effects of estrogens acting at multiple levels could provide a sophisticated means of modulating a variety of functions, including pain sensitivity.

This study did not directly investigate endogenous spinal EM2 antinociception. However, we expect that current findings likely pertain to regulating endogenous spinal EM2 analgesic functionality since some spinal neurons coexpressing MOR, mER α , mGluR $_1$ and aromatase are apposed by EM2 varicosities. Although the gene encoding endomorphins has not been found, (1) the human proteome contains two proteins with the sequence of a Gly-extended

EM2 that are flanked by Gly-Ser cleavage sites [61], (2) Tyr-Pro, has been proposed to serve as a biosynthetic precursor for EM [86], and (3) the presence of EM1/EM2 in the CNS was demonstrated biochemically via Edman degradation and sequencing, independent of antibody recognition [104].

The relevance of current findings to nociception/opioid antinociception in women remains to be established. Notably, the peak severity of many chronic pain conditions predominantly afflicting women (e.g., fibromyalgia, migraine) occurs around the time of menses [39], which is comparable to diestrus in rats. Physiological suppression of endogenous spinal EM2 antinociception at specific menstrual cycle stages and the inability to transition out of that suppressed state, could contribute to the greater frequency and severity of chronic pain syndromes in women than men.

The mechanisms by which estrogens influence nociception/opioid antinociception is a gateway to understanding how nociceptive stimuli are processed and modulated in females. The current demonstration that aromatase oligomerizes with mERs and other plasma membrane receptors, enabling spatially restricted activation of mERs to be inversely related to circulating levels of estrogens, can explain why some estrogen-dependent influences on nociception/opioid antinociception are in-phase and others out-of-phase with ovarian estrogen production. Additionally, our finding that MOR functions within an oligomer containing regulators of its activation could inform the development of novel pharmacotherapies that target interactions among oligomer components, not only for chronic pain management (in women), but also for other CNS disorders resulting from impaired estrogenic signaling.

5. Conclusion

The current study reports a new modality of estrogenic signaling in spinal cord. We show the association of aromatase with the plasma membrane receptors mER α , mGluR₁ and MOR, allowing estrogens to function as intra-neuronal messengers whose synthesis and actions are segregated within a plasma membrane signaling unit that regulate spinal EM2 antinociception. This novel organization of aromatase with mERs explains why some estrogen-dependent functionalities are in-phase and others out-of-phase with circulating estrogen levels. The reported oligomerization of ERs could inform therapeutic interventions for chronic pain syndromes disproportionately afflicting women.

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Figure 1.

The robust i.t. EM2 antinociception manifest during proestrus is not altered by blockade of mER α , GPR30 or mER β . Spinal antinociception was quantified using the tail flick latency test. I.t ER blockers or their vehicle (Veh=DMSO) followed by EM2 (45 nmol) was applied at the indicated times. Data are presented as mean $\pm$ SEM tail flick latency (TFL). n=4–8. MPP (10 nmol, ER α blocker); G15=G-15 (10 nmol, GPR30 blocker); PHT=PHTPP (10 nmol, ER β blocker).

Figure 2.

During diestrus, blockade of spinal mERs or inhibition of spinal aromatase unmasks i.t. EM2 antinociception. Spinal antinociception was quantified using the tail flick latency test. I.t. EM2 (45 nmol) or its vehicle was administered at the indicated times. Data are presented as mean±SEM tail flick latency (TFL). **A.** Spinal EM2 antinociception during diestrus was manifest 30 min following blockade of ER α (via i.t. MPP, 10 nmol) or GPR30 (via i.t. G-15, 10 nmol), but not ER β (via i.t. PHTPP, 10 nmol). Vehicle for ER blockers (Veh1=DMSO) had no effect on TFL. $n=4-8$. G15=G-15, PHT=PHTPP. Inset shows the ability of MPP to restore spinal EM2 analgesic responsiveness when administered 5 min

preceding i.t. EM2 (10 min prior to analgesic testing). n=4. **B.** Spinal analgesic responsiveness to EM2 during diestrus that is unmasked by spinal ER α blockade retains the dose response characteristics of i.t. EM2 in proestrous rats. I.t. EM2 (15 or 45 nmol) or its 3% DMSO vehicle (Veh2) was administered 30 min after i.t. MPP (10 nmol), or its DMSO vehicle. Inset shows spinal EM2 antinociceptive responsiveness to 15, 45 or 90 nmol (5 min after i.t. application; peak effect) during diestrus (D) vs. proestrus (P). n=4–8. **C.** Inhibition of spinal aromatase via i.t. fadrozole (2.5 nmol, 1 h), but not its vehicle (Veh1), during diestrus unmasks spinal EM2 antinociception. n=4–5. FAD=fadrozole.

Figure 3.

mERα and GPR30 co-IP with aromatase. Solubilized spinal cord membrane fractions were IPed using an antibody selective for aromatase ($\oplus$). **A:** IPs were Western blotted using antibodies selective for either ERα or GPR30. Both ERs (required for suppression of spinal EM2 antinociception during diestrus) co-IPed with aromatase from spinal cord of diestrous rats. The magnitude of co-IP did not differ from that obtained from spinal cord of proestrous rats, data not shown. Specificity of the ERα and GPR30 Western signals is shown by their absence when Western blotting with preadsorbed antibody. **B:** Specificity of anti-aromatase antibodies. Aromatase was IPed from spinal cord using anti-aromatase ($\oplus$) but not its preadsorbed control ($\emptyset$). IPs were Western blotted as described in Methods using antibodies generated against an epitope different from that used to generate the IP antibody. The ≈ 50 kDa signals visualized in Western blots of aromatase IP using anti-aromatase antibody (+; lane 1) were virtually eliminated when blotting with preadsorbed antibody (-; lane 2). Lane 3 shows the absence of the Western signal when blotting IP obtained using preadsorbed anti-aromatase IP antibody ($\emptyset$). Aro= aromatase; WB=Western blot; (+) denotes presence of primary antibody; (-) denotes preadsorbed primary antibody.

Figure 4.

Activity of aromatase-mER α is elevated during diestrus vs. proestrus. **A.** Aromatase IP, obtained as in Fig. 3, was Western blotted using an antibody selective for phosphoSer118ER α (pSER α). **B.** Bar graph shows quantification of differences in signal intensity of the $\approx$ 35 and $\approx$ 50 kDa pSER α molecular mass species normalized with respect to IPed aromatase. The augmented signal intensity of pSER α in diestrus, indicating increased ER α activation, likely reflects increased activity of the associated aromatase during diestrus. $\ast=p<0.05$ for comparison of co-IPed pSER α from spinal cord of D (diestrus) vs. P (proestrus) rats. **C.** Specificity of anti-pSER α antibody. The antigen used to generate the antibody against pSER α (pS-pep) and its nonphosphorylated congener (non-pS-pep) (11 aa surrounding Ser118; 2 μ g each) were Western blotted using anti-pSER α antibody. Whereas a Western signal was obtained when blotting the phosphorylated antigenic peptide, no signal was obtained when blotting its nonphosphorylated congener. Aro=aromatase; WB=Western blot.

Figure 5. mGluR₁ is required for suppression of spinal EM2 analgesia during diestrus. Spinal antinociception was quantified using the tail flick test during diestrus at the indicated times. I.t. EM2 or its vehicle was administered 30 min following either the blockade of spinal mGluR₁ (via 25 nmol YM298198), mGluR_{2/3} (via 25 nmol LY341495) or their vehicle. During diestrus, blockade of spinal mGluR₁, but not mGluR_{2/3}, rapidly revealed a robust spinal analgesic response to i.t. EM2. Data are presented as mean±SEM tail flick latency; n=4–8. YM= YM298198; LY= LY341495; Veh1=DMSO vehicle; Veh2=3% DMSO vehicle.

Figure 6.

Association of spinal mER α and mGluR $_1$ is greater during diestrus than proestrus. IP was obtained from solubilized spinal membranes of diestrous and proestrous rats using anti-mGluR $_1$ antibody. mER α present in mGluR $_1$ IP was assessed via Western analysis using anti-ER α antibody. **A.** mGluR $_1$ IP contained ≈ 35 and ≈ 50 kDa species of ER α . **B.** Bar graph shows quantification of differences in signal intensity of each molecular mass species, normalized with IPed mGluR $_1$. The content of both ER α species (mean $\pm$ SEM; n=6) was significantly greater in mGluR $_1$ IP obtained from spinal cord of diestrus vs. proestrus rats. *= $p < 0.05$ for comparison of co-IPed ER α from spinal cord of D (diestrous) vs. P (proestrous) rats. **C.** Specificity of anti-mGluR $_1$ antibodies. mGluR $_1$ was IPed from spinal cord using anti mGluR $_1$ antibody ($\oplus$) but not its preadsorbed control ($\emptyset$). IPs were Western blotted as described in Methods using antibodies generated against an epitope different from that used to generate the IP antibody. The ≈ 137 kDa signal visualized in Western blots of mGluR $_1$ IP using anti-mGluR $_1$ antibody (+; lane 1) was eliminated when blotting with the preadsorbed antibody (-; lane 2). Lane 3 shows the absence of the Western signal when blotting IP obtained using preadsorbed anti-mGluR $_1$ IP antibody ($\emptyset$). WB=Western blot.

Figure 7.

Spinal aromatase is present in an oligomer containing mER α , mGluR₁ and MOR. A: Solubilized spinal cord membranes were electrophoresed using Blue native (BN) gels. A ≈ 360 kDa band was identified via Western blotting (WB) using anti-MOR antibody. B: The ≈ 360 kDa band identified in A was stained with Coomassie blue and eluted. Reducing SDS-PAGE Western analysis of the eluate revealed the presence of aromatase (Aro), ER α , mGluR₁ and MOR. C: Solubilized spinal membranes were subjected to sequential IP using (in order) antibody against aromatase, ER α , and mGluR₁. The final IP was electrophoresed using BN gels and Western blotted with anti-MOR antibody, which also revealed the ≈ 360

kDa band. Our results cross-validate that aromatase, mER α , mGluR₁ and MOR are present in a presumptive signaling complex in spinal cord.

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Figure 8.

Neurons coexpressing MOR, mGluR₁, aromatase and ERα are apposed by EM2 in the superficial dorsal horn of the L6 spinal segment in diestrus rats. **A** (top panels): Aromatase, ERα and mGluR₁ and MOR are coexpressed in a single neuron in the superficial dorsal horn of segment L6 during diestrus. Images show single optical sections from two adjacent 5 μm physical sections through the same neuron. **LEFT**: Aromatase-ir (green; arrows) and mGluR₁-ir (red; arrowheads) are expressed in or near MOR-ir (blue) in the plasma membrane. (Overlay of green and blue is cyan; overlay of red and blue is magenta.) Larger "spearhead" on bottom-right shows MOR-ir, aromatase-ir and mGluR₁-ir expressed in the

same region. *Insets* shows 2× enlarged images of triple labeling in the latter region. **RIGHT:** The same MOR-ir (blue) neuron expresses ERα-ir (green) in its cell body and both ERα-ir (arrow) and mGluR₁-ir (red; arrowheads) in or near the plasma membrane. **B** (bottom panels): EM2 apposes a neuron expressing MOR, ERα and mGluR₁. Images are of two adjacent 5 μm physical sections of a single MOR-ir neuron in L6 superficial dorsal horn. Blue=MOR-ir; red=mGluR₁-ir; green=EM2-ir (left) or ERα-ir (right). **LEFT:** MOR-ir neuron expresses mGluR₁ in or near the plasma membrane (arrowhead). Its dendrite, extending to the right, is apposed by two EM2-ir varicosities (arrows). Image is a projection of 4 consecutive optical sections (2 μm total distance in the z-axis). Overlay of green and blue produces cyan. **RIGHT:** The same MOR-ir neuron expresses ERα-ir in or near its plasma membrane (arrows) and within its cell soma. These observations suggest the presence of an anatomical organization in spinal cord that is consistent with our hypothesis that spinal EM2 antinociception results, at least in part, from its innervating spinal neurons that both synthesize and respond to estrogen.

Figure 9.

Interactions among components of an oligomer containing aromatase (Aro), mERs, mGluR₁ and MOR underlie estrous cycle-dependent analgesic responsiveness to i.t. EM2. During diestrus, estrogens (E) synthesized within this oligomer (denoted by the green oval), activate mER/mGluR₁ signaling, thereby suppressing EM2 antinociception. During proestrus, interactions between mGluR₁ and mERs that suppress EM2 antinociception are lost (denoted by the broken yellow line between mGluR₁ and mER). We envision that this loss results from the physical dissociation of mGluR₁ and mERs, and/or intra-molecular rearrangement of oligomer components.