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Title: Pleiotropic opioid regulation of spinal endomorphin 2 release and its adaptations to opioid withdrawal are sexually dimorphic

Sumita Chakrabarti**,** Nai-Jiang Liu, James E. Zadina, Tarak Sharma and Alan R Gintzler

Department of Obstetrics and Gynecology, State University of New York, Downstate Medical Center, Brooklyn, New York (SC, NJL**,** TS, ARG), SE LA Veterans HCS, Departments of Medicine, Pharmacology and Neuroscience, Tulane Univ Sch of Medicine, New Orleans, LA (JEZ)

Corresponding Author: Alan Gintzler
SUNY Downstate Medical Center
450 Clarkson Ave
Brooklyn, NY 11203

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Abstract

We studied adaptations to acute precipitated opioid withdrawal of spinal mu-opioid receptor (MOR)-coupled regulation of the release of endomorphin 2 (EM2). The release of this highly MOR-selective endogenous opioid from opioid naïve spinal tissue of male rats is subjected to MOR-coupled positive as well as negative modulation via cholera toxin-sensitive G_s and pertussis toxin-sensitive G_i/G_o, respectively. The net effect of this concomitant bidirectional modulation is inhibitory. MOR-coupled pleiotropic regulation of EM2 release is retained in opioid withdrawn spinal tissue of male rats but the balance of MOR-coupled inhibitory and facilitatory regulation shifted such that facilitatory regulation predominates. Augmented coupling of MOR to G_s is causally associated with this change. Strikingly, pleiotropic characteristics of MOR-coupled regulation of spinal EM2 release and adaptations thereof to opioid withdrawal are male-specific. In females, however, MOR-coupled regulation of EM2 release from opioid naïve and withdrawn spinal tissue does not have a significant G_s coupled facilitatory component and MOR-coupled inhibition of EM2 release persists unabated in withdrawn preparations. The male specific adaptations to chronic morphine that shift the relative predominance of opposing dual G protein-coupled MOR pathways provides a mechanism for mitigating inhibitory MOR signaling without losing MOR-coupled feedback regulation. These adaptations enable utilizing endogenous EM2 as a substitute for morphine that had been precipitously removed. The sexually dimorphic functionality and regulation of spinal EM2/MOR–coupled signaling suggest the clinical utility of using sex-specific treatments for addiction that harness the activity of endogenous opioids.

Introduction

The interplay between exogenously administered opioids and endogenous opioid systems remains largely unaddressed. Many studies have investigated the effects of morphine on endogenous opioid peptides, opioid receptor gene expression (Uhl et al., 1988; Brodsky et al., 1995; Romualdi et al., 1995; Ronnekleiv et al., 1996; Fang et al., 1998; Yeh et al., 1998), and opioid receptor trafficking (Whistler et al., 1999; Cahill et al., 2001; Eisinger et al., 2002). Nevertheless, the relevance of tolerance mechanisms recruited by exogenous opiates, e.g., morphine, to the functionality of endogenous opioid systems has not been elucidated. This could provide a window into the physiological recruitment of endogenous opioids to mitigate withdrawal sequelae.

Effects of acute and chronic administration of morphine, the most commonly used opiate, are predominantly mediated via the μ-opioid receptor (MOR), for which there are several endogenous substrates. Of these, endomorphin (EM) 1 and 2 not only have the highest affinity and selectivity for MOR over the δ-opioid receptor (DOR) and κ-opioid receptor (KOR) but are also potent endogenous antinociceptive substrates (Zadina et al., 1997). We selected EM2 for the current study since it is the predominant spinal EM (Martin-Schild et al., 1998) and its release not only exhibits sexual dimorphism, e.g., magnitude, modulation by ovarian sex steroids, but is also subjected to MOR-coupled regulation (Gupta et al., 2007).

Activation of spinal MOR inhibits evoked spinal EM2 release whereas neither spinal KOR nor DOR activation does so (Gupta et al., 2007). Moreover, *in vitro* blockade of spinal opioid receptors *augments* basal as well as evoked release of spinal EM2 (Gupta et al., 2007). These data indicate that release of spinal EM2 is subject to MOR-coupled negative feedback (Gupta et al., 2007), and underscore the importance of tonic *endogenous* MOR-coupled pre-synaptic inhibition to regulating spinal EM2 release. The reciprocal relationship between spinal MOR

activation and spinal EM2 release makes it an ideal system for exploring adaptations to chronic morphine in a system that utilizes MOR-coupled regulation to maintain homeostasis.

Chronic morphine elicits multiple cellular adaptations. In addition to adaptations that result in diminished MOR functionality (Sim et al., 1996), we have identified multiple cellular adaptations that shift acute MOR-coupled adenylyl cyclase signaling from inhibitory to stimulatory (Chakrabarti et al., 1998; Rivera and Gintzler, 1998; Chakrabarti et al., 2001; Chakrabarti and Gintzler, 2003; Chakrabarti et al., 2005a; Chakrabarti et al., 2005b; Chakrabarti and Gintzler, 2007). These post receptor adaptations would be particularly advantageous to neurons utilizing EM2 given their role not only in mitigating the persistent inhibition of EM2 release imposed by the sustained presence of morphine but also in preserving MOR-coupled regulation of that release, which would be eliminated by downregulation/uncoupling of opioid receptors.

This study was designed to test the hypothesis that withdrawal from chronic systemic opioid treatment, which is sufficient to induce analgesic tolerance, elicits adaptive cellular mechanisms that utilize the pleiotropy inherent in receptor G protein coupling to maintain MOR-coupled regulation of EM2 release. We also investigated the sex dependency of these adaptations since spinal morphine recruits sexually dimorphic antinociceptive mechanisms (Liu et al., 2007; Chakrabarti et al., 2010). Using acute *in vitro* withdrawal to reflect changes that had occurred during chronic systemic morphine exposure, our findings indicate that chronic morphine shifts MOR-coupled regulation of EM2 release from inhibitory to stimulatory, which results from augmented coupling of MOR to G_s ($G_s\alpha$). Notably, these adaptations occur in males, but not females. These findings provide physiological relevance for the previously reported chronic morphine induced alteration of MOR G protein coupling and suggest that the recruitment of endogenous EM2 to cope with opioid withdrawal is sexually dimorphic.

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Materials and Methods

Experimental animals. Sprague-Dawley male and female rats (250-300g; Charles River, Kingston, NY), employed in experiments, were maintained in an approved controlled environment with food and water *ad libitum*. All surgeries were performed under sodium pentobarbital (Anpro pharmaceuticals, Arcadia, CA) anesthesia (40 and 50 mg/kg, i.p., in females and males, respectively) and all experimental procedures were reviewed and approved by the Animal care and use committee of the SUNY Downstate Medical Center.

Implantation of Intrathecal Cannulae. A permanent indwelling cannula was inserted into the lumbar spinal cord subarachnoid space as utilized previously (Liu et al., 2011). Briefly, in anesthetized animals, a saline filled catheter (PE-10, Clay Adams) was introduced through a small incision in the atlanto-occipital membrane, slowly inserted into the subarachnoid space 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. All animals appeared to be free of infection upon gross inspection. Integrity of the motor system was assessed in all groups using the righting reflex and the inclined plane test. Those exhibiting motor impairment following surgery were eliminated from the study.

Spinal Application of Pertussis Toxin and Cholera toxin. Bacterial toxin(s), Pertussis toxin (PTX) and/or Cholera toxin (CTX) (List Biologicals, Campbell, CA, for both), were injected via the intrathecal cannula to block G_i/G_o and G_s proteins, respectively. PTX (10 μg/5 μl) was injected every other day (day1, day3 and day5) during the last five days before sacrifice. CTX (5 μg/5 μl) was injected once 24 hours prior to sacrifice.

Systemic administration of Morphine. Morphine or placebo were administered via the subcutaneous implantation of morphine base or placebo pellets (Cicero and Meyer, 1973) (one pellet on day 1, two pellets on day 3 and three pellets on day 5, six pellets in total; each

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morphine pellet contained 75 mg morphine base). The gradual escalating dosing of systemic morphine is analogous to many medicinal settings where increasing doses of morphine are required for severe unremitting and/or escalating pain. On day 7, animals were sacrificed according to institutional guidelines by decapitation, after which cervical through lumbar spinal tissue was quickly expelled by injecting ice-cold saline into the rostral end. Spinal cord obtained from animals treated chronically with morphine and equilibrated for 40 min *in vitro* in the absence of morphine was used to model opioid withdrawal**.** Since experiments were designed to test adaptations to opioid withdrawal of the MOR, we determined effects elicited by sufentanil (a highly MOR-selective alkaloid) instead of morphine (which activates DOR and KOR as well as MOR). Furthermore, epidural sufentanil or fentanyl is frequently employed for medicinal purposes.

Spinal Tissue Preparation for Superfusion. The spinal vertebral column was sectioned at the intervertebral spaces above vertebrae T12 and below L5. The lumbar spinal cord contained within this segment (L1–L5; 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., Guilford, 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_2PO4, 25 mM NaHCO3, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11.1 mM dextrose, and gelatin (saturated with 4 g/l) and was gassed with a 95% O_2/5% CO_2. Additionally, the Krebs' superfusate used to assess basal and stimulated EM2 release contained the protease inhibitors captopril (10 μM), thiorphan (0.3 μM), bestatin (10 μM), and L-leucyl-L-leucine (2 mM) to protect peptides against the degradation resulting from the actions of the tissue proteases (Gupta et al., 2007).

Superfusion Paradigm. The basal release of EM2 was defined as EM2 content in spinal superfusate obtained over 18 min (6 ml) in the absence of high potassium. The magnitude of

evoked EM2 release was determined over a 3-min period (1.8 ml) by quantifying the content of EM2 in spinal superfusate that contained high potassium (K^+; 50 mM; the content of sodium was proportionally reduced to maintain osmolarity). This constituted the first cycle of release. Following stimulation with high K^+, a 15-min rest period ensued before re-determining basal and assessing evoked EM2 release in the presence of sufentanil (μ-opioid receptor agonist), or naloxone (opioid receptor antagonist) (1 μM). In all cases, the magnitude of K^+-evoked release in the presence of drug (cycle 2) was compared with the magnitude of release observed in its absence (cycle 1). Determination of EM2 release in the presence of selected pharmacological agents constituted the second release cycle. Basal release [B] was subtracted from the total release while in the presence of high K^+, stimulated release [S], to calculate the increment in evoked release [S-B] The percent increment in evoked release=[(S-B)/B] x100, which was determined in absence (cycle 1) and presence (cycle 2) of drug. A third cycle of release was obtained in which the conditions of cycle 1 were repeated to ensure that changes in basal and/or stimulated release of EM2 were attributable to the presence of drug and not simply to the passage of time. Drug effects were always reversed following washout and re-equilibration (cycle 3). Basal and stimulated superfusate were collected into pre-chilled tubes on ice. Superfusate containing basal release and evoked release tubes were desalted and concentrated using reverse phase C-18 cartridges (Sep-Pak; Waters Corp. Milford, MA). EM2 peptide was eluted, lyophilized to dryness and stored at 4°C until quantified using radioimmunoassay (RIA).

EM2 Quantification using RIA. EM2 was assayed using RIA which employed a rabbit antibody (1:10,000) raised against EM2 (a generous gift from Dr. James Zadina) and highly specific for this peptide (Martin-Schild et al., 1997). Samples were incubated with anti-EM2 antibody (1:10,000; 2h; room temp.) prior to adding radiolabeled ^{125}I-EM2 (specific activity 2203 Ci/mmol; Phoenix Pharmaceuticals, Burlingame, CA), which was incubated overnight. Antibody

bound radioactivity was determined using scintillation proximity (Gupta et al., 2007). The reaction mixture was transferred from tubes to 96 well scintillation impregnated plates that were coated with anti-rabbit antibodies. The radiolabeled antigen-antibody complexes bound to secondary antibody were in proximity to scintillation bound to the plates and were counted. In contrast, the non-bound ^{125}I-EM2 did not come into proximity with the secondary antibody and scintillation and was not counted. The plates were counted in a Microbeta plate reader (PerkinElmer, Boston, MA). A standard curve was generated (0.5-16 pg/tube) in which percent inhibition of binding was plotted against the log concentrations of non-labeled peptide. Values from samples were calculated from the standard curve generated with non-labeled EM2 using 'Forecast function' of Microsoft Excel.

Membrane preparation and Immunoprecipitation. Spinal cord membranes were prepared and solubilized as described previously (Chakrabarti et al., 2005a). Briefly, animals were sacrificed and spinal cord tissues were isolated as described before. Tissues were homogenized in 20 mM HEPES (pH 7.4) containing 10% sucrose, 5 mM EDTA, 1 mM EGTA, 2 mM dithiothreitol (DTT), 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-*p*-Tosyl-L-phenylalanine chloromethyl ketone, N_{α}-Tosyl-L-lysine chloromethyl ketone and phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO) and complete cocktail inhibitor tablet/50 ml (Roche Molecular Biochemicals, Indianapolis, IN). Supernatants from a low-speed spin (1,000 g for 10 min) were centrifuged at a higher speed spin (30,000 g) for 40 min to obtain membrane pellets. Immunoprecipitates were obtained from membranes solubilized in the above buffer containing 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% Na-deoxycholate, 0.1% Na-dodecyl sulfate and 10% glycerol, agitated 60 min at 4°C and centrifuged (16,000 g for 40 min at 4°C). $G_s\alpha$ was immunoprecipitated as described previously (Chakrabarti et al., 2005a). Briefly, immunoprecipitation (ip) was initiated with 50 μg of

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solubilized membrane and a rabbit polyclonal antibody generated against the C-terminus of the $G_s\alpha$ subunit (aa 385-394; BD Biosciences Pharmingen, San Diego, CA). Pre-washed Protein A-agarose (50 µl; Roche Molecular Biologicals, Indianapolis, IN) was used for immunoprecipitation overnight at 4°C. The beads were washed in 20 mM HEPES buffer (pH 7.4) containing 2 mM DTT, 5mM EDTA, 150 mM NaCl, 0.05% NP-40 and the same protease inhibitors as mentioned above. Immunoprecipitates were eluted by heating the samples in 30 µl of sample buffer (15 min at 85°C). Samples separated on 4-12% gradient Bis-Tris gels (Invitrogen) were electro-transferred onto nitrocellulose membranes and used for Western analyses.

Western analysis. Standard procedures were used for Western analyses as utilized previously (Chakrabarti et al., 2005a). $G_s\alpha$ protein was visualized using a 1:10,000 dilution of a rabbit polyclonal anti- $G_s\alpha$ antibody generated against the C-terminus of $G_s\alpha$ (generous gift from Dr. John Hildebrandt, Medical University of South Carolina). MOR protein was visualized using a 1:8,000 dilution of a rabbit polyclonal antibody generated against the C-terminal 50 aa of MOR (Chalecka-Franaszek et al., 2000) (generously provided by Dr. Thomas Cote, Uniformed Services University of the Health Sciences, Bethesda, Maryland). The secondary antibody utilized was a peroxidase-labeled donkey anti-rabbit antibody (1:20,000; GE Healthcare). Antibody-substrate complex was visualized using Supersignal West Dura Chemiluminescence detection kit (Pierce, Rockford, IL). Specificity of Western signals was demonstrated previously via their diminution/elimination following incubation with antibodies while in the presence of a 3-5-fold excess of their respective blocking peptides (Chakrabarti et al., 2005a). Sample pairs, obtained from opioid naïve and chronic morphine-withdrawal spinal cords were processed, electrophoresed and blotted in parallel after which they were exposed concomitantly to a GBox (CCD camera; Syngene, Frederick, MD). Intensity of signals was quantified by using Genetools software (Syngene).

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Statistical Analysis. Kruskal-Wallis and Mann Whitney nonparametric tests were used to assess significance of differences among groups. Significance of within group differences was assessed using Student's t test.

Results

Opioid withdrawal shifts sufentanil modulation of evoked EM2 release from inhibition to facilitation. Fig. 1A illustrates that sufentanil (100 nM) inhibited K^+-evoked EM2 release from spinal cord of opioid naïve rats (-54 ± 9%; n=3, $p<0.05$), as previously reported from this laboratory (Gupta et al., 2007). In contrast, the same concentration of sufentanil stimulated K^+-evoked EM2 release (167±11%; n=3, $p<0.02$) from spinal cord obtained from chronic morphine treated male rats that had been equilibrated *in vitro* in the absence of morphine (withdrawn, spinal cord) (Fig. 1A). Statistical analyses confirmed apparent differences between sufentanil effects in opioid naïve vs. withdrawn spinal tissues (Fig. 1A, bar 1 vs. bar 2; $p<0.05$). Qualitative changes in MOR-coupled modulation of EM2 release from *in vitro* withdrawn spinal tissue was not evident from similarly treated spinal tissue obtained from female rats (see below).

Dose dependence of sufentanil facilitation of evoked spinal EM2 release during opioid withdrawal. Fig. 1B illustrates that sufentanil stimulation of K^+-evoked EM2 release from withdrawn spinal tissue of males was observed at all concentrations tested (1-1000 nM). Strikingly, however, the magnitude of sufentanil stimulation of EM2 release was not dose dependent (Fig. 1B; 252-233%; $p>0.05$; n=3-5 for each concentration). Since MOR can couple to G_s as well as G_i/G_o G proteins, the proportion of which is influenced by chronic morphine (Chakrabarti et al., 2005a; Chakrabarti and Gintzler, 2007), we investigated whether the apparent lack of sufentanil dose dependency resulted from the concomitant activation of opposing MOR-coupled G proteins by determining sufentanil dose responsiveness after chemically uncoupling spinal MOR from G_i/G_o.

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PTX treatment unmasked a significant dose dependency of sufentanil facilitation of EM2 release from withdrawn spinal tissues of male rats ($p<0.05$). Intrathecal PTX not only unmasked dose dependency of sufentanil stimulation of evoked EM2 release from withdrawn spinal tissue (267-505%; n=3; Fig. 1C) but also substantially increased the magnitude of that stimulation [505% (Fig. 1C) vs 167% (Fig. 1A) for with and without PTX treatment, respectively, at 100 nM sufentanil; $p<0.05$]. This indicates that stimulatory and inhibitory MOR signaling can concomitantly regulate EM2 release from *in vitro* withdrawn spinal tissue but the net effect of MOR activation in these preparations is facilitation. Importantly, facilitation by sufentanil (100 nM) of EM2 release from withdrawn spinal tissues was completely abolished by the intrathecal pretreatment with CTX in addition to PTX, strongly suggesting that sufentanil facilitation of evoked EM2 release from withdrawn spinal tissue is mediated via G_s (Fig. 1C; $p<0.01$; n=4).

MOR G_s interaction is augmented in opioid withdrawn spinal tissues of males. The co-immunoprecipitation (co-ip) of MOR with $G_s\alpha$ was used to reflect changes in their interaction, as previously reported by this laboratory (Chakrabarti et al., 2005a; Chakrabarti and Gintzler, 2007). Sufentanil (1 μM) did not produce any detectable increase in the MOR content of $G_s\alpha$ immunoprecipitate obtained from opioid naïve spinal tissues. In contrast, the same concentration of sufentanil increased 5-fold ($p<0.05$; n=3) the MOR content of $G_s\alpha$ immunoprecipitate obtained from withdrawn spinal tissues (Fig. 1D, lane 4 vs. 3; n=3). Importantly, the withdrawal-associated increment in MOR $G_s\alpha$ co-ip was completely abolished by the addition of the opioid receptor antagonist naloxone concomitant with sufentanil (Fig. 1D, lane 5 vs. lane 4; n=2), indicating that the ability of sufentanil to increase MOR $G_s\alpha$ co-ip was opioid receptor-mediated.

To validate the inferred causal association between the emergent sufentanil facilitation of K^+-evoked EM2 release and the augmented MOR G_s coupling in withdrawn spinal tissue, we investigated the dose dependency and CTX reversibility of sufentanil stimulation of MOR $G_s\alpha$

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co-ip. Sufentanil dose dependently increased MOR G_s co-ip (Fig. 1E and 1F; 80-572%; n=3-6), paralleling its ability to facilitate evoked EM2 release. The sufentanil-stimulated increase in G_s-MOR interaction in withdrawn spinal cord was also abolished following intrathecal treatment with CTX (Fig. 1E, lane 6 vs. lane 5 and Fig 1F, bar 5 vs bar 4, n=3). These data, in combination with those described above, underscore the relevance of enhanced MOR G_s signaling to the emergent predominance of CTX-sensitive sufentanil facilitatory modulation of EM2 release that occurs in opioid withdrawn spinal tissue of males.

Inhibition of protein phosphatase 2A (PP2A) blocks both the withdrawal-induced emergence of sufentanil facilitation of evoked EM2 release and augmented MOR-G_s coupling. We previously demonstrated that dephosphorylation of $G_s\alpha$ by PP2A is essential for the enhanced MOR-G_s interaction in spinal cord membranes obtained from chronic morphine treated rats (Chakrabarti et al., 2007). To further validate the role of MOR G_s signaling in the switch from MOR-coupled inhibition to facilitation of evoked EM2 release, we investigated the effect of inhibiting spinal PP2A on the withdrawal-associated emergence of sufentanil facilitation of evoked EM2 release and MOR G_s coupling. Intrathecal administration of the PP2A inhibitor Calyculin A (25 nM) not only abolished sufentanil facilitation of evoked EM2 release (Fig. 2A, n=3) but also eliminated sufentanil stimulated MOR $G_s\alpha$ co-ip (Fig. 2B; n=3) from withdrawn spinal tissue of males.

Opioid withdrawal enhances G_s-coupled MOR regulation of EM2 release that is present in spinal cord of opioid naïve male rats. The switch from MOR-coupled inhibition to facilitation of EM2 release from opioid withdrawn spinal cord could result from either the chronic morphine-induced *de novo* emergence of a novel MOR-coupled signaling pathway or the amplification of a pathway that is present but masked in opioid naïve tissue. In order to distinguish between these possibilities, we investigated whether or not intrathecal PTX unmasked facilitation by sufentanil of EM2 release from opioid naïve spinal tissue. Intrathecal

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PTX did unmask the presence of a MOR-coupled facilitation of EM2 release from opioid naïve spinal tissue (Fig. 3; compare 68±9% inhibition vs. 135±34% facilitation by 1 μM sufentanil without and with PTX, respectively; n=3; Fig. 3, bars 2 vs 1), which was abolished following intrathecal treatment with CTX concomitant with PTX (Fig. 3, bars 3 vs 2). This indicates that MOR G_s-coupled regulation of EM2 release is indeed present in opioid naive spinal tissue. However, it is present at a greatly reduced level [compare enhanced EM2 release produced by 100 nM (76%; data not shown) or 1 μM sufentanil (120%, Fig. 3) with the 505% increment produced by 100 nM sufentanil in withdrawn tissue (Fig. 1C)].

Opioid withdrawal-induced switch from inhibitory to facilitatory MOR-coupled modulation of evoked EM2 release is sexually dimorphic. In contrast to opioid withdrawn spinal tissue of males, 1 μM sufentanil continued to inhibit evoked EM2 release from withdrawn spinal tissue of females (Fig. 4A, bar 3; -66±24%; n=3). Moreover, although intrathecal PTX essentially abolished the inhibition of EM2 release produced by 1 μM sufentanil, PTX did not unmask any facilitation of evoked EM2 release (Fig. 4A, bar 4; n=3), as was observed in withdrawn tissues of males (compare Fig. 4A, bar 2 with Fig. 1C).

Pleiotropic MOR-coupled regulation of evoked EM2 release from opioid naïve spinal tissue is sexually dimorphic. MOR activation by 1 μM sufentanil inhibited evoked EM2 release from opioid naïve spinal tissue of females (Fig. 4A, bar 1; -63±6%; n=3), as we had previously reported (Gupta et al., 2007). This inhibition, as expected, was essentially eliminated by intrathecal PTX (Fig. 4A, bar 2, n=3). However, intrathecal PTX did not unmask any sufentanil facilitation of evoked EM2 release, as occurred in similarly treated spinal tissue of males (compare Fig. 4A, bar 2 with Fig. 3, bar 2). Notably, neither the magnitude of sufentanil inhibition of EM2 release nor the effects thereon of intrathecal PTX differed between opioid naïve and withdrawn spinal tissue of females (compare Fig. 4A, bars 1 and 3 and bars 2 and 4, respectively; $p>0.05$ for both comparisons).

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Sufentanil fails to augment MOR G_s association in withdrawn spinal tissue of females. In contrast to the sufentanil-induced enhancement of MOR-G_s association in withdrawn spinal tissue from male rats, sufentanil failed to stimulate MOR-G_s association in corresponding tissue from females (Fig. 4B; lane 2 vs 1, $p>0.05$). These results are in keeping with the absence of qualitative changes in MOR-coupled regulation of evoked EM2 release from withdrawn spinal tissues of females.

Opioid withdrawal shifts endogenous MOR-coupled regulation of spinal EM2 release from inhibitory to facilitatory in males, but not females. Naloxone (1 μM) enhances the evoked release of EM2 from the spinal tissue of opioid naïve males as well as females (Gupta et al., 2007), presumably reflecting blockade of the inhibitory effects of endogenous opioids on MOR autoreceptors. Strikingly, however, the same treatment with naloxone inhibits evoked EM2 release from withdrawn spinal tissue of males (Fig. 5, bar 2), presumably reflecting the blockade of facilitatory, G_s-mediated, effects of endogenous opioids on MOR autoreceptors. In contrast to these observations, opioid withdrawal failed to reverse the direction of sufentanil or naloxone modulation in the spinal cord of females (Fig. 5). In withdrawn spinal tissue of females, sufentanil continued to inhibit (Fig. 5 bar 3) and naloxone continued to facilitate evoked release of EM2 (Fig. 5, bar 4; 31.5%; n=3; $p<0.05$). Thus, in both males and females, MOR-coupled endogenous regulation of spinal EM2 release parallels its regulation by sufentanil (Fig. 5, bars 1 and 3, respectively). These findings with naloxone also support the concept of a shift in coupling from G_i/G_o to G_s in males, but not in females.

Discussion

Results of this study reveal that sexually dimorphic spinal adaptations are elicited by acute opioid withdrawal despite its largely sexually monomorphic behavioral presentation.

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Additionally, utilizing the same preparation to study biochemical and functional sequelae of opioid withdrawal enabled assessment of the physiological implications of specific cellular adaptations.

Specific findings in males include: (1) evoked EM2 release from opioid naïve and withdrawn spinal cord is concomitantly subjected to negative as well as positive MOR-coupled modulation; (2) in opioid withdrawn spinal tissue, the balance of MOR-coupled regulation of EM2 release shifts from predominantly inhibitory to facilitatory; (3) the qualitative shift in MOR-coupled regulation of EM2 release results from augmented MOR G_s coupling; (4) MOR-coupled regulation of spinal EM2 release and changes thereof following opioid withdrawal exhibits striking sexual dimorphism. In females, MOR-coupled regulation of EM2 release from opioid naïve spinal tissue does not exhibit bimodal MOR-coupled regulation; opioid withdrawal neither augments MOR-coupled facilitation of EM2 release nor MOR G_s coupling. MOR-coupled regulation of spinal EM2 release in males exhibits physiological state-dependency that is not manifest in the spinal cord of females.

Pleiotropic bidirectional MOR-coupled regulation of EM2 release is masked in the spinal cord of opioid naïve males. This is revealed by the ability of intrathecal PTX to not only eliminate inhibition of evoked EM2 release by sufentanil but to also unmask its ability to facilitate that release. Neither inhibition nor facilitation was observed following intrathecal PTX and CTX.

Abolition by CTX of the facilitatory sufentanil modulation strongly suggests G_s-mediation. Thus, it was surprising that we were not able to detect increased MOR in $G_s\alpha$ ip following acute sufentanil. This apparent dichotomy most likely results from much greater sensitivity of the EM2 release mechanism to small increases in $G_s\alpha$ vs. the ability of the Western method employed to detect them. Additionally, the CTX sensitivity of sufentanil facilitated EM2 release utilized PTX-treated tissue, which could have facilitated detecting MOR G_s coupling, whereas MOR $G_s\alpha$ co-ip was investigated in the absence of PTX.

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The newly emerged predominance of MOR-coupled facilitatory regulation of EM2 release from opioid withdrawn spinal tissues of males did not exhibit any sufentanil dose dependency over three orders of magnitude. However, intrathecal PTX not only dramatically augmented the magnitude of facilitatory regulation of EM2 release by sufentanil, but also unmasked its dose dependency, all of which was eliminated by intrathecal CTX. The parsimonious interpretation of these data is that sufentanil concomitantly activates MOR G_s- and MOR G_i/G_o-coupled regulation of EM2 release, the relative contributions of which do not significantly vary over the range of sufentanil concentrations employed.

The effects of intrathecal PTX and CTX underscore that pleiotropic MOR-coupled modulation of EM2 release that was present in opioid naïve spinal tissue from males persisted in opioid withdrawn spinal tissue but the balance between MOR-coupled inhibition and facilitation shifted. Whereas in opioid naïve preparations the net effect of the bidirectional MOR-coupled control was inhibitory, facilitatory MOR-coupled modulation predominated in opioid withdrawn tissue. This plasticity of MOR-coupled bidirectional modulation of EM2 release enables the sensory MOR system to accommodate changing physiological demand.

In withdrawn spinal cord of males, cross validating biochemical and pharmacological observations indicate that augmented MOR G_s coupling is causally associated with the switch from MOR-coupled inhibition to facilitation of EM2 release. (1) Intrathecal CTX eliminated the facilitation of evoked EM2 release by sufentanil. (2) Sufentanil stimulation of MOR G_s association (reflected by MOR $G_s\alpha$ co-ip) was dramatically elevated in opioid withdrawn spinal tissue. (3) Stimulation of MOR G_s association by sufentanil was dose-dependent, paralleling the sufentanil facilitation of evoked EM2 release that was manifest after eliminating contributions of G_i/G_o activation. (4) Naloxone eliminated sufentanil stimulation of MOR G_s coupling indicating that it was triggered by activation of MOR. This conclusion is supported by the high selectivity of sufentanil for MOR and by the inability of KOR and DOR to modulate evoked EM2 release (Gupta et al., 2007). (5) Inhibition of spinal PP2A (via calyculin), which blocks $G_s\alpha$

dephosphorylation that is essential for chronic morphine-induced augmented MOR G_s coupling (Chakrabarti and Gintzler, 2007), eliminated the withdrawal-induced emergence of sufentanil facilitation of evoked EM2 release as well as augmented MOR-G_s coupling.

We had previously reported that chronic morphine increases MOR G_s coupling in cell lines as well as spinal cord (Chakrabarti et al., 2005a; Chakrabarti and Gintzler, 2007; Shy et al., 2008). Present results reaffirm those findings and put them into a physiological context, i.e., enhanced MOR G_s signaling during withdrawal enables opioid facilitation of EM2 release, which in turn contributes to withdrawal coping mechanisms. The demonstrated importance of adaptations in MOR G_s signaling to the predominance of MOR-coupled facilitatory regulation of EM2 release in withdrawn spinal cord does not eliminate the possibility that the qualitative shift in MOR regulation of EM2 release is multifactorial, i.e., it could also result from additional putative sources such as alterations in MOR G_i/G_o signaling. It should be noted, however, that PTX would not be expected to have the profound effect that it did on sufentanil facilitatory modulation of EM2 release (Fig. 1C) if the G_i-coupled MOR that is relevant to spinal EM2 release was substantially uncoupled during withdrawal.

Of critical relevance to the *in vivo* regulation of spinal EM2 release during opioid withdrawal in males, the shift from negative to positive opioid regulation of EM2 release manifested in response to sufentanil is also evident in response to an endogenously released opioid(s). We previously reported (Gupta et al., 2007), and confirmed in the present study, that spinal opioid receptor blockade in naïve males, enhances evoked EM2 release. However, opioid receptor blockade inhibited evoked EM2 release from opioid withdrawn spinal tissues of males. This indicates that under opioid naïve conditions, spinal EM2 release is subject to ongoing endogenous negative feedback, but during withdrawal, EM2 release is subjected to positive feedback. Co-localization of MOR and EM2 in fibers of the outer laminae of the spinal dorsal horn (Martin-Schild et al., 1997; Abbadie et al., 2002; Aicher et al., 2003) provides an anatomical basis for MOR-coupled regulation of spinal EM2 release.

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Strikingly, MOR-coupled dual regulation of spinal EM2 release as well as its adaptation to opioid withdrawal is sexually dimorphic. MOR-coupled inhibition of evoked EM2 release was comparable in spinal cord of males and females. However, in contrast to the emergence of MOR-coupled facilitation of evoked EM2 release from withdrawn spinal tissue of males, MOR-coupled inhibition of evoked EM2 release from withdrawn spinal tissue of females persisted unabated and it did not revert to a facilitation even following intrathecal PTX. Consistent with these observations, naloxone modulation of evoked EM2 release was not qualitatively different between opioid naïve vs. opioid withdrawn spinal preparations obtained from females.

Regulation of spinal EM2 release by EM2/MOR-mediated feedback inhibition (Gupta et al., 2007) indicates that spinal synaptic levels of EM2 are very tightly regulated by a closed loop in which MOR functions as an EM2 sensor. This places particular restrictions on the types of adaptive mechanisms to chronic morphine that would be advantageous to spinal EM2-transmitting neurons. If negative MOR G_i/G_o-coupled modulation of spinal EM2 release persisted during long-term systemic morphine administration, spinal EM2 utilization would be greatly suppressed. MOR desensitization (uncoupling) would eliminate or substantially reduce the continual opioid inhibition of release but this adaptation would also eliminate EM2 feedback regulation of its own release (which is MOR-coupled), an important regulatory parameter. Thus, there is an imperative for additional or alternative adaptations to chronic morphine.

Adaptations (in males) to chronic morphine that involve shifting the relative predominance of opposing dual G protein-coupled MOR pathways that are concomitantly activated provides a mechanism for mitigating inhibitory MOR signaling without losing MOR-coupled feedback regulation. Moreover, a shift in the equilibrium from predominantly MOR G_i/G_o negative to MOR G_s positive modulation of EM2 release would have a much greater dynamic range than would just uncoupling MOR G_i/G_o inhibitory signaling since the magnitude of any increased EM2 utilization resulting from disinhibition (uncoupling) alone would be limited by the extent of ongoing presynaptic inhibition.

The male-specific enhanced utilization of the spinal EM2/MOR-coupled system during opioid withdrawal is consistent with other reports of MOR-related sexual dimorphism, e.g., (1) greater antinociceptive responsiveness to morphine of male vs. female rats (Cicero et al., 1996; Cicero et al., 1997; Wang et al., 2006; Loyd et al., 2008), (2) greater K^+-induced release of EM2 from spinal cord of male vs. female rats (Gupta et al., 2007), (3) exclusive mediation of spinal morphine antinociception by spinal MOR in males but KOR as well as MOR in females (Liu et al., 2007) and (4) the almost 5-fold greater expression of MOR heterodimerized with KOR in the spinal cord of females than males (Chakrabarti et al., 2010). All suggest the predominance and perhaps the exclusivity of MOR-mediated events in males vs. the importance in females of alternative opioid systems, alone or in combination with MOR.

Sexually dimorphic adaptations of the spinal EM2 system to chronic morphine indicate that males and females differentially utilize endogenous spinal EM2 to cope with the precipitous removal of morphine following its chronic systemic administration. In withdrawn spinal cord of males, augmented MOR G_s signaling and the resultant increase in EM2 release enable utilizing endogenous EM2 to substitute for morphine that had been precipitously removed. The absence of this adaptation in the spinal cord of females suggests that alternative coping strategies, yet to be determined, are employed.

The demonstrated sexually dimorphic spinal EM2/MOR–coupled signaling suggests the clinical utility of using sex-specific treatments for addiction that harness the activity of endogenous opioids. The predominance of MOR G_s-coupled facilitative modulation of EM2 release from withdrawn spinal tissue of males, but not females, could suggest that clinical management of opioid dependence in women would benefit (more than in men) from adjunctive pharmacotherapies that enhance spinal EM2 release.

Authorship contribution

Sumita Chakrabarti: participated in research design; conducted experiments; performed data analysis; wrote or contributed to the writing of the manuscript

Nai-Jiang Liu: participated in research design; conducted experiments; performed data analysis; wrote or contributed to the writing of the manuscript

James Zadina: performed data analysis; contributed new reagents or analytic tools; wrote or contributed to the writing of the manuscript

Tarak Sharma: participated in research design; conducted experiments; performed data analysis; wrote or contributed to the writing of the manuscript

Alan Gintzler: participated in research design; performed data analysis; wrote or contributed to the writing of the manuscript

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Legends for Figures

Figure 1. In spinal cord of male rats, opioid withdrawal augments MOR-coupled facilitation of evoked EM2 release and MOR coupling to G_s. A, Sufentanil inhibited EM2 release from naïve spinal tissue (Nv, bar 1). In contrast, in withdrawn spinal tissue, sufentanil enhanced evoked EM2 release (WD, bar 2), n=3-4. %Δ represents the difference of the percent increment in K^+-evoked EM2 release, calculated as [(evoked-basal) / basal] x100, in the absence and presence of sufentanil. B, Sufentanil (1-1000 nM) enhances evoked EM2 release from opioid withdrawn spinal tissues but this modulation was not dose dependent (bars 1-4), n=3-5. C, Intrathecal PTX unmasks the dose-dependence of the facilitation by sufentanil (bars 1-3; 1-100 nM; n=3), which was abolished by pretreatment of intrathecal CTX in addition to PTX (bar 4; n=3). D illustrates MOR Western analysis of immunoprecipitates obtained from opioid naïve (lanes 1,2) and withdrawn (lanes 3-5) spinal cord using anti-$G_s\alpha$ antibodies. Immunoprecipitation was performed in the absence (lanes 1,3) and presence (lanes 2,4,5) of 1 μM sufentanil. Sufentanil stimulation of the co-ip of MOR with $G_s\alpha$ was abolished by naloxone (1 μM; lane 5). Lanes 1,2 and 3-5 were processed and blotted in parallel. E, Dose-dependent stimulation by sufentanil of MOR in $G_s\alpha$ immunoprecipitate (lane 1-5) and its abolishment by intrathecal CTX (lane 6). All lanes were processed and blotted in parallel. F illustrates quantification of the sufentanil-stimulated increment in MOR $G_s\alpha$ co-ip illustrated in E (n=3). Opioid withdrawal augments facilitatory MOR-coupled modulation of EM2 release, which is causally associated with increased coupling of MOR with G_s; †p<0.05 for withdrawal effect; #p<0.05 for sufentanil dose effect; *p<0.05 for CTX effect.

Figure 2. Inhibition of PP2A activity blocks both the stimulation of evoked EM2 release by sufentanil and its enhancement of MOR $G_s\alpha$ coupling in opioid withdrawn spinal tissue of males. A. Intrathecal pretreatment with the PP2A inhibitor, Calyculin A (caly) abolished the sufentanil (1

μM) stimulation of evoked EM2 release from opioid withdrawn spinal tissue of males (WD + caly; n=3; p<0.01). Sufentanil (1 μM) stimulation of evoked EM2 release (WD) is replicated from Fig. 1B to facilitate comparison. B, Co-immunoprecipitation of MOR with $G_s\alpha$ was determined in parallel from withdrawn spinal tissue in the absence (lane 1) and following (lane 2) intrathecal treatment with calyculin A (n=3). Inhibition of spinal PP2A abrogated both the withdrawal-associated augmented facilitatory modulation by sufentanil as well as the enhanced coupling of MOR to G_s.

Figure 3. MOR-coupled regulation of EM2 release from the spinal cord of opioid naïve male rats exhibits pleiotropy. Inhibition of evoked EM2 release by sufentanil (1 μM; bar 1) reverses to a facilitation following intrathecal PTX (bar 2, *=p<0.05), all of which was eliminated by concomitant intrathecal treatment with PTX and CTX (compare bar 3 vs. bar 2, #= p<0.05), n=3 for all experimental groups. Results indicate that facilitatory MOR-coupled modulation of spinal EM2 release is present in opioid naïve spinal cord, albeit at a greatly reduced level.

Figure 4. Absence of augmented MOR-coupled facilitation of evoked EM2 release and MOR G_s coupling in opioid withdrawn spinal cord of females. A. In females, the magnitude of sufentanil (1 μM) inhibition of EM2 release from opioid naïve spinal tissue (bar 1) was comparable to that observed from opioid withdrawn spinal preparations (bar 3). Intrathecal PTX essentially abolished the inhibition by sufentanil of EM2 release from both opioid naïve and withdrawn preparations (bars 2 and 4, respectively; * p< 0.05 for effect of PTX in naïve; # p< 0.05 for effect of PTX in withdrawn tissue; n=3 for both). B. MOR Western analysis of $G_s\alpha$ immunoprecipitate obtained from opioid withdrawn spinal tissue in the absence (lane 1) or presence (lane 2) of 1 μM sufentanil. Neither opioid naïve nor withdrawn spinal tissue of females manifests facilitatory MOR-coupled modulation of EM2 release. Enhanced MOR G_s coupling was also absent in withdrawn tissue of females.

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Figure 5. Endogenous MOR-coupled regulation of evoked EM2 release shifts from inhibitory to facilitatory in withdrawn spinal tissue of males, but not females. *In vitro* MOR blockade via naloxone (1 μM) inhibits evoked EM2 release from withdrawn spinal tissue of males (bar 2) whereas similar treatment enhances evoked EM2 release from withdrawn spinal tissue from females (bar 4), n=3 for both. Effects of 1 μM sufentanil on evoked EM2 release from withdrawn spinal tissue of males (bar 1) and) females (bar 3) were reproduced from Fig 1B and Fig 4A, respectively, to facilitate comparison with the directionality of effects of naloxone. In withdrawn tissues of both males and females, modulation of EM2 release by naloxone was inversely proportional to effects of MOR activation by sufentanil. Enhancement vs. inhibition of evoked EM2 release by MOR blockade in withdrawn spinal cord of males and females, respectively, reveals the predominance of endogenous MOR-coupled positive modulation of spinal EM2 release in males but the persistence of negative modulation in spinal cord of females.

Fig. 1

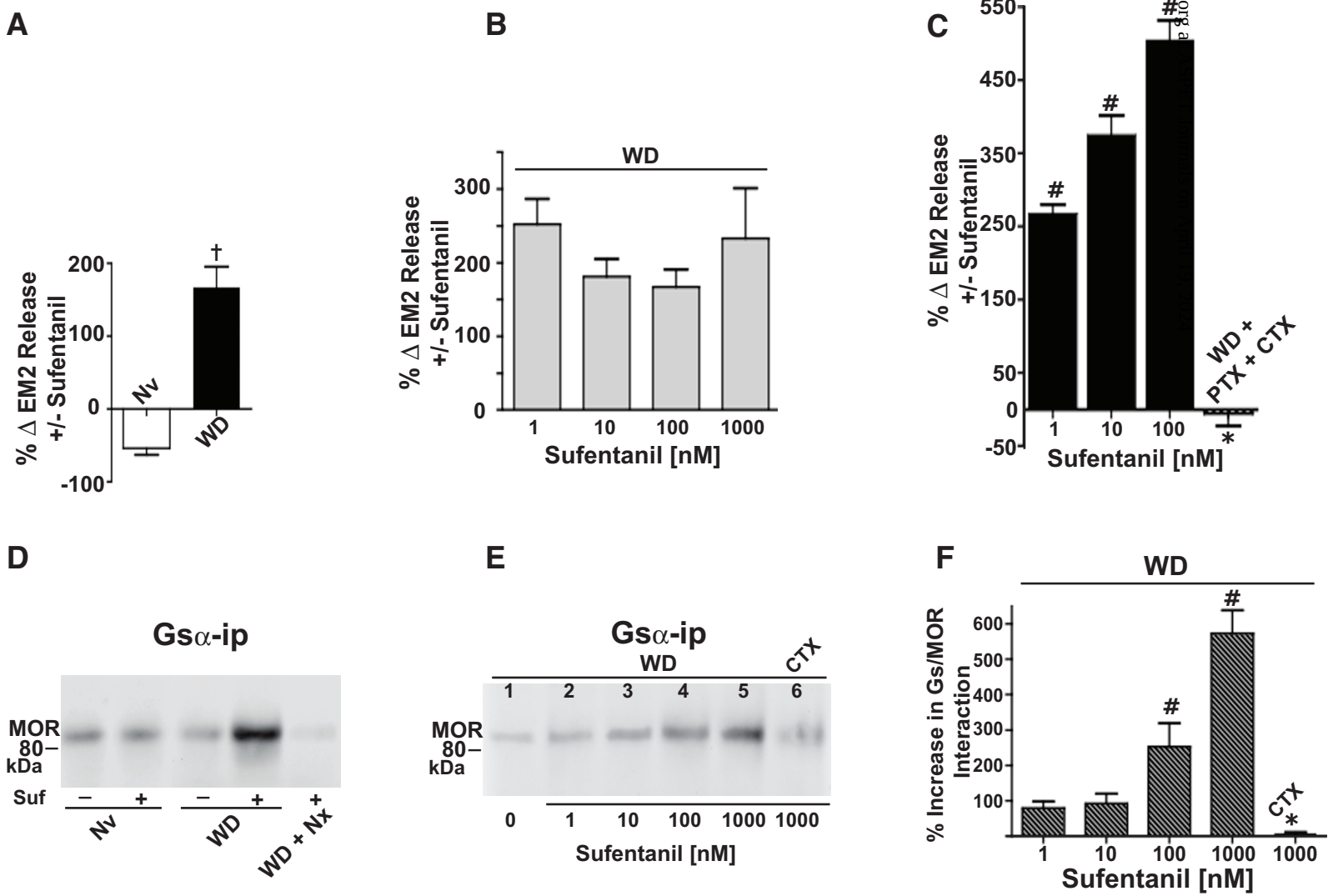

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Fig. 2

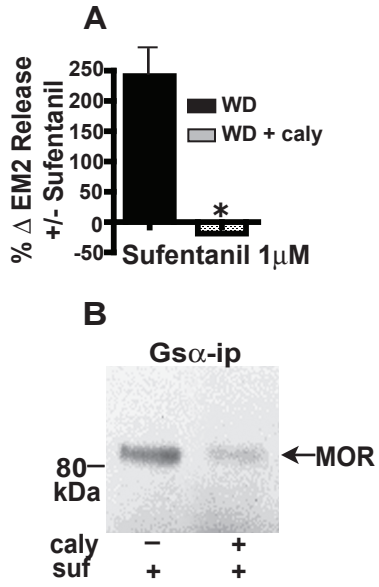


Fig. 3

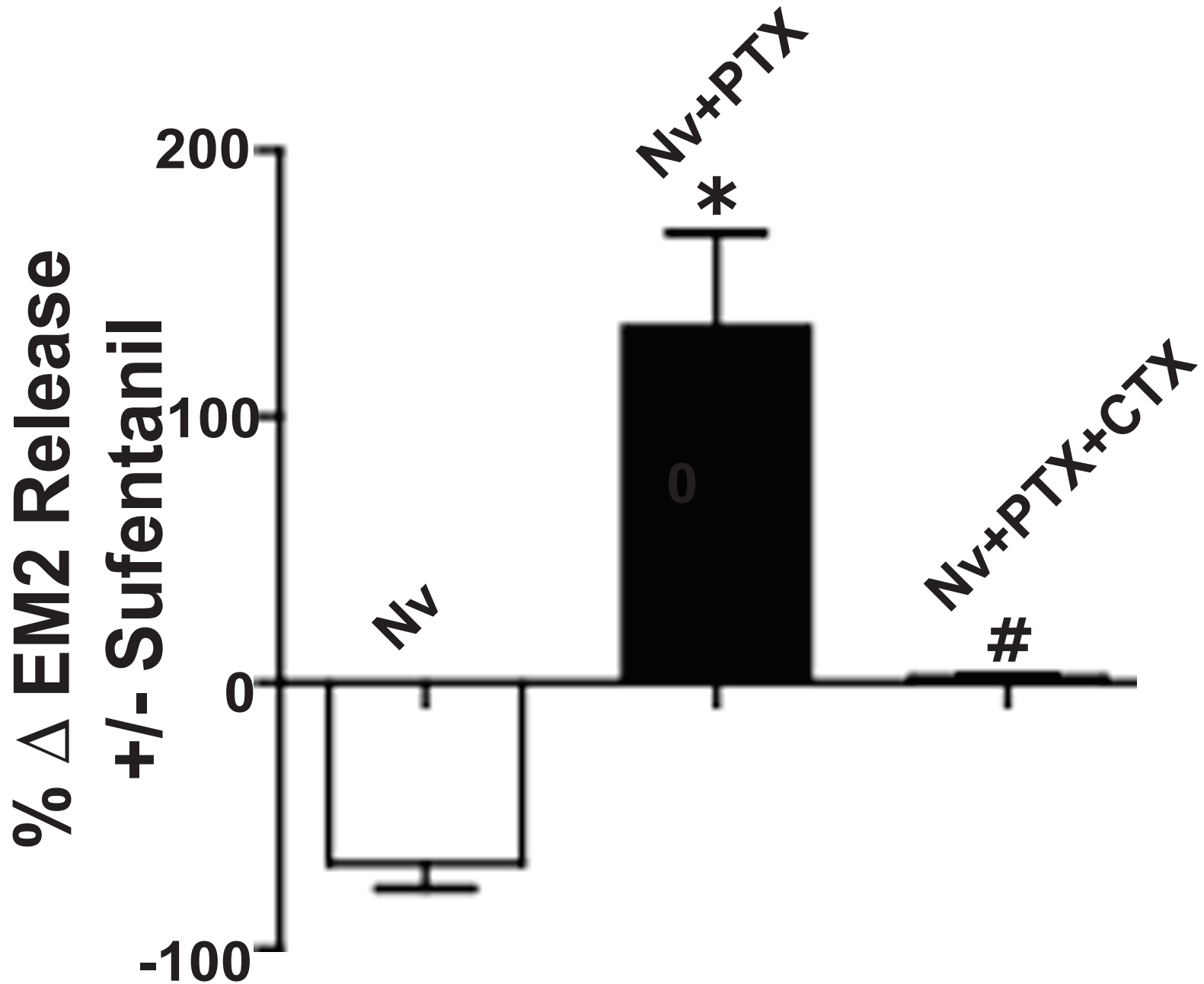
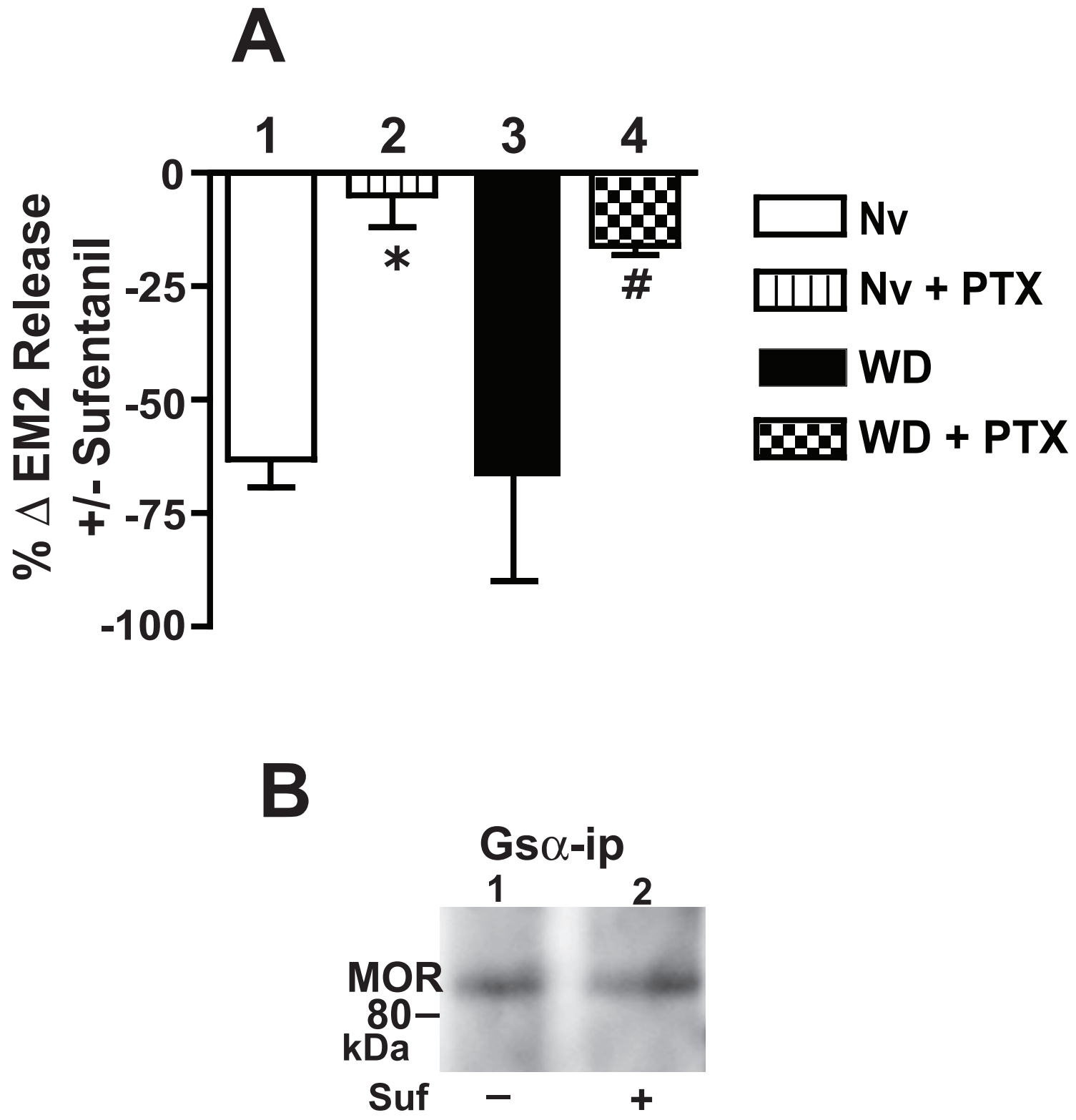


Fig. 4


A
1
2
3
4
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-25
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-75
-100
% Δ EM2 Release
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*
#
Nv
Nv + PTX
WD
WD + PTX
B
Gsα-ip
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2
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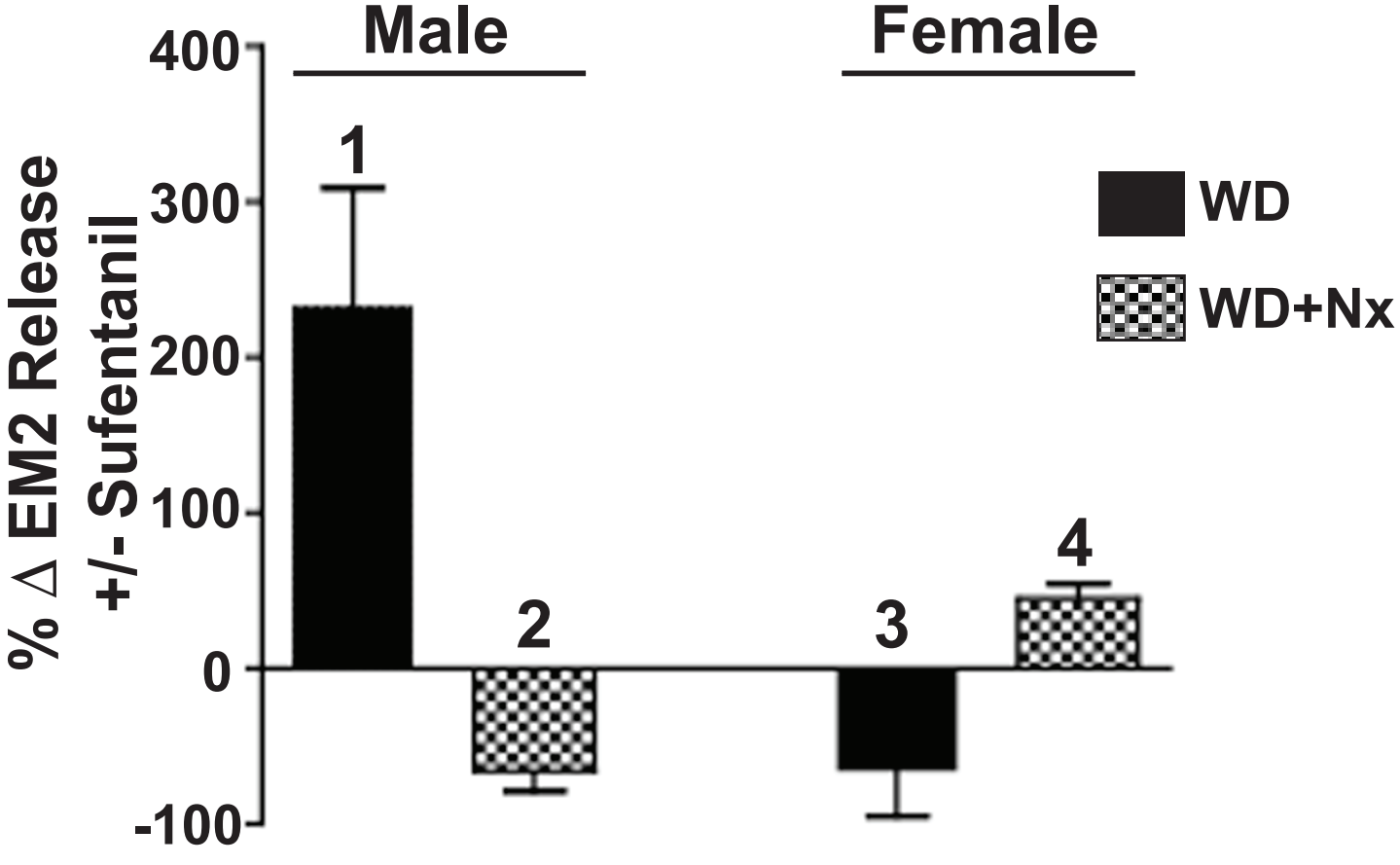
Fig. 5


Male
Female
400
300
200
100
0
-100
% Δ EM2 Release
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1
2
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4
WD
WD+Nx