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Mu-opioid receptor splice variants: Sex-dependent regulation by chronic morphine

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Abstract

The gene encoding the mu-opioid receptor (MOR) generates a remarkable diversity of subtypes, the functional significance of which remains largely unknown. The structure of MOR could be a critical determinant of MOR functionality and its adaptations to chronic morphine exposure. Since MOR antinociception has sexually dimorphic dimensions, we determined the influence of sex, stage of estrus cycle and chronic systemic morphine on levels of MOR splice variant mRNA in rat spinal cord. Chronic systemic morphine influenced the spinal expression of mRNA encoding rMOR-1B2 and rMOR-1C1 in a profoundly sex-dependent fashion. In males, chronic morphine resulted in a 2-fold increase in expression levels of rMOR-1B2 and rMOR-1C1 mRNA. This effect of chronic morphine was completely absent in females. Increased density of MOR protein in spinal cord of males accompanied the chronic morphine-induced increase in MOR variant mRNA, suggesting that it reflected an increase in corresponding receptor protein. These results suggest that tolerance/dependence results, at least in part, from different adaptational strategies in males and females. The signaling consequences of the unique composition of the C-terminus tip of rMOR-1C1 and rMOR-1B2 could point the way to defining the molecular components of sex-dependent tolerance and withdrawal mechanisms.

Keywords

mu-opioid receptor; splice variants; sexual dimorphism; opioid tolerance

Introduction

OPRM1, the only identified gene encoding the mu-opioid receptor (MOR), (Chen *et al.* 1993) generates a remarkable diversity of MOR subtypes (Pasternak 2001). Most are C-terminal variants that are generated through alternative splicing between exon 3 and multiple downstream exons (Pasternak & Pan 2013). The discovery of these and other splice variants (e.g., exon 11 variants with extensive and 5' splicing, exon 1 variants containing a single

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transmembrane domain) provide a molecular biology framework for the concept of multiple mu-opioid receptors inferred from much earlier pharmacological studies (Wolozin & Pasternak 1981).

Some functional differences have been identified among MOR splice variants. Many opioids show marked differences in efficacy and potency at C-terminal MOR variants (Pasternak *et al.* 2004, Pan *et al.* 2005b, Bolan *et al.* 2004, Pan *et al.* 2005a). Morphine-induced internalization also varies among the C-terminal variants, e.g., morphine given intracerebroventricularly internalizes mMOR-1C1 but not mMOR-1 (Abbadie & Pasternak 2001).

Differences among MOR splice variants also vary with respect to agonist selectivity. Knockout of exon 1 abolishes morphine analgesia, but not that of either morphine-6β-glucuronide or heroin (Schuller *et al.* 1999). Conversely, knockout of exon 11 greatly reduces analgesic responsiveness to morphine-6β-glucuronide and heroin, but not to morphine or methadone (Pan *et al.* 2009). MOR transcripts also vary in their expression patterns and levels of expression across brain regions (Abbadie *et al.* 2000b, Abbadie *et al.* 2000c), implying region-specific processing, and differing physiological functions. MOR splice variants have also been implicated to explain incomplete cross-tolerance among agonists and variability of agonist potency/effectiveness in patients.

Strikingly, MOR splice variants have not been studied with respect to the well-documented sexual dimorphism in MOR antinociception in laboratory animals (Barrett *et al.* 2002, Cicero *et al.* 1997, Cicero *et al.* 1996, Boyer *et al.* 1998, Krzanowska & Bodnar 1999, Cook *et al.* 2000, Peckham & Traynor 2006) and, in humans, (Sarton *et al.* 2000). This laboratory has recently reported two striking manifestations of sexually dimorphic properties of MOR in spinal cord: (1) in opioid naïve spinal tissue the propensity of MOR to heterodimerize with the kappa opioid receptor (KOR) is substantially greater in females than males, the magnitude of which is dependent on stage of estrus cycle, i.e., spinal levels of the MOR/KOR heterodimer during proestrus are significantly greater than during diestrus (Chakrabarti *et al.* 2010, Liu *et al.* 2011) and (2), chronic systemic morphine treatment augments MOR G_s coupling in the spinal cord of males but not females (Chakrabarti *et al.* 2012).

The structure of MOR could be a determinant of both the facility with which spinal MOR heterodimerizes with KOR as well as the G proteins with which MOR preferentially couples. Accordingly, we investigated the influence of stage of estrus cycle and chronic systemic morphine (in both males and females) on spinal cord levels of mRNA encoding MOR splice variants. We selected MOR variants representing each of the two main classes of splicing that have been identified in rodents and humans. For example, MOR-1C1 and MOR-1B2 represent variants resulting from extensive 3’ splicing at the end of the C-terminus but containing the seven transmembrane spanning domains and the N-terminus of MOR-1. MOR-1G1 represents variants resulting from extensive 5’ splicing containing six transmembrane spanning domains and an altered N-terminus.

Results reveal a striking sexual dimorphism in the ability of chronic systemic morphine to selectively influence the generation of spinal MOR variants. Implications for sex-based opioid tolerance mechanisms are discussed.

Materials and methods

Animals

Sprague-Dawley rats (Charles River, Kingston, NY; 225–300 g) were maintained in an approved controlled environment on a 12 h light/dark cycle. Food and water were available ad libitum. Experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of SUNY Downstate Medical Center.

Determination of stage of estrous cycle

Stage of cycle was determined using histology of vaginal smears. Predominance of small leukocytes was indicative of diestrus; a predominance of large round nucleated cells was indicative of proestrus.

Systemic administration of morphine

Morphine was administered via the subcutaneous implantation of morphine base pellets (75 mg each) (Chakrabarti et al. 2012, Villar & Bhargava 1992). On day 7, animals were sacrificed by decapitation. Lumbar spinal tissue was expelled by injecting ice-cold saline into the rostral end of the spinal cord and either immediately submerged in RNA Later (Ambion) solution for RNA extraction or ice-cold 50 mM Tris buffer (pH 7.4) for radioligand binding studies.

Tissue preparation for RNA extraction

Transverse sections of whole lumbar spinal cord were homogenized in a Teflon glass homogenizer in Trizol reagent. Homogenates were spun (3,000g, 5 min), supernatants were mixed with 0.2 ml of chloroform (incubated for 3 min, room temperature), after which they were centrifuged at 12,000g for 15 minutes at 4°C. The aqueous phase was precipitated with 0.5 ml of isopropanol. Resulting pellets of total RNA were washed twice in 75% ethanol and either stored in 75% ethanol (−80°C) or resuspended in nuclease free water (130 ng/μl) for immediate use. Aliquots (260 ng; 2 μl) of each sample were used for RT-PCR. Every sample was analyzed in an Agilent Bioanalyzer to evaluate the quality of the RNA and quantify its concentration. Samples with RNA Integrity numbers less than seven were discarded.

RT-PCR

A Step One Plus apparatus (Applied Biosystems, Life technologies, Grand Island, NY) was programmed to run the following reverse transcriptase real time PCR (RT-PCR) scheme: 7 min at 42°C (retro transcription); 10 s at 95°C (denaturation). PCR amplification was achieved by 40 cycles of the following: 5 s at 95°C, 10 s at 58°C, 4 s, at 72°C. Amplicon dissociation was achieved by 15 s at 95°C, 1 min at 60°C, and 15 s at 95°C. Reactions were performed using TAKARA One Step SYBR® Ex Taq ™ qRT-PCR Kit (Takara Bio Inc., Madison, WI), containing SYBR RT-PCR mix (12.5μl), Taq polymerase 2.5U (0.5 μl),

retrotranscriptase mix (0.5 μl), forward primer (0.2 μM, 0.5 μl), reverse primer (0.2 μM, 0.5 μl), RNAse free water (8.5 μl) and total RNA (260 ng; 2 μl).

All oligonucleotides used for quantitative RT-PCR produced amplicons ranging from 100 bp to 250 bp. Each was designed and tested against a rat database for possible unwanted products by using the Primer Blast online software: The following were the primers employed: rMOR-1/rMOR-1G1: f-GAACAGCAAAACTCCACTCGAG; r-GCAATGGAGCAGTTTCTGCCT; rMOR-1A: f-AGGGAACATCCCTCCACGGCT; r-GGACTTTGCCTCAAGTTACTGACCTT; rMOR-1B2: f-CCAACCTCGTCCACGATCGAACA; r-TGTCTCTACTGACTGAGGCTCCTGGT; rMOR-1C1: f-ACGTGGTCTTTGAAATCGCGTGC; r-ACTTCTCACTAGCACATCCCTGTGT; rMOR-1G1; f-TCCGCATTCCAAAAACTGGACAGG; r-ACTGGTCGCTAAGGCGTCTGC; rMOR-1R; f-AGGGAACATCCCTCCACGGCTAATA; r-ACCTACCATTGTGGGGGCCAAG. All RT-PCR reactions including negative controls were run in duplicates.

The number of cycles required for each amplicon to reach detection, via interaction with SYBR green, was defined as the threshold cycle (C_T). The C_T for each of the six MOR variants studied and glucose-6-phosphate dehydrogenase (G6PD) was concomitantly determined from spinal cord of opioid naïve, diestrus, proestrus female and male rats. G6PD was used as both a reference internal control gene to normalize the RT-PCRs for the amount of RNA added to the reactions and to calculate a relative mRNA value for the splice variants of interest, which was calculated as $2^{-\Delta CT}$ where (ΔC_T) is the difference between the C_T for each MOR variant and G6PD (Livak & Schmittgen 2001).

To assess the influence of chronic morphine on MOR variant expression, we first calculated the difference between variant-specific mRNA levels in spinal cord of opioid naïve and chronic morphine-treated rats (ΔC_T), which was expressed relative to the ΔC_T of the variant mRNA of the lowest abundance (rMOR-1R), ($\Delta\Delta C_T$). Expression levels of rMOR-1R were not influenced by chronic morphine. MOR variant mRNA doubles with each PCR cycle at the exponential phase of a PCR reaction, when the C_T is recorded. Since C_T is inversely proportional to mRNA levels, the relative expression of specific MOR variants in naïve vs. opioid tolerant spinal cord was calculated as $2^{-\Delta\Delta CT}$ (Livak & Schmittgen 2001).

Radioligand binding studies

Receptor number (B_{max}) and K_D estimates before and following chronic systemic morphine treatment were determined by saturation binding using [^{3}H]DAMGO. Lumbar spinal tissue obtained from two placebo- or chronic morphine-treated rats were pooled, homogenized in 50 mM Tris buffer (pH 7.4) and centrifuged at 40,000g for 30 min at 4°C. Pellets were resuspended in Tris buffer, incubated (30 min, 25°C) and centrifuged again (40,000g, 30 min). Resulting pellets, were resuspended in 50 mM potassium phosphate buffer (pH 7.2) for duplicate determination of [^{3}H]DAMGO binding (0.1 -10 nM; 25°C, 90 min; ≈100 μg protein/tube). Assays were terminated by adding ice-cold buffer and filtration over Whatman GF/B filters. Filter-bound radioactivity was detected using liquid scintillation spectrophotometry. Nonspecific binding was determined in the presence of 10 μM naltrexone.

Statistical analysis

One-way ANOVA followed by Tukey's multiple comparison test were used to compare mRNA levels among groups. Nonlinear regression analysis was used to estimate B_{max} and K_D derived from saturation binding studies. All binding data were best fit by a one-site model.

Results

Expression levels of spinal MOR splice variants in spinal tissue obtained from naïve proestrus and diestrus female and male rats

The relative abundance of spinal cord mRNA ($2^{-\Delta CT}$; see methods) encoding rMOR-1, rMOR-1A, rMOR-1B2, rMOR-1C1, rMOR-1G1 and rMOR-1R was assessed with RT-PCR using the mRNA encoding G6PD as the internal reference. One-way ANOVA followed by Tukey's multiple comparison test were used to analyze data pertaining to each MOR variant. Table 1 illustrates that levels of mRNA encoding rMOR-1 (the most abundant variant mRNA), rMOR-1A, rMOR-1C1, and rMOR-1R did not differ among any of the groups. However, levels of mRNA encoding rMOR-1B2 and rMOR-1G1 was significantly (~3-fold) greater in the spinal cord of diestrus females vs. males ($F_{2,15}$ =8.44 and $F_{2,12}$=4.875, respectively, $P<0.05$ for both, Table 1). These differences notwithstanding, spinal cord levels of rMOR-1B2 and rMOR-1G1 did not significantly differ between diestrus and proestrus or proestrus vs. male rats.

Chronic morphine differentially influences the abundance of specific spinal MOR splice variants in a sex-dependent fashion

Since spinal cord of opioid naïve proestrus and diestrus female rats did not differ in the content of mRNA encoding those MOR splice variants under study and stage of estrus cycle is disrupted by chronic morphine (Craft *et al.* 1999), stage of estrus cycle was not considered as a separate factor in investigations of sex-dependent effects of chronic systemic morphine on spinal MOR variant mRNA expression.

Chronic systemic morphine influenced the spinal expression of some MOR variants in a sex-dependent fashion (Fig 1). In the spinal cord of males, chronic morphine increased expression levels ≈2-fold of mRNA encoding rMOR-1B2 and rMOR-1C1 (ANOVA; $F_{5,30}$=6.636, $p<0.05$ for both rMOR-1B2/rMOR-1C1; n=5–8) (Fig. 1). However, in striking contrast to males, in females, chronic morphine failed to alter spinal levels of mRNA encoding rMOR-1B2 and rMOR-1C1. Notably, spinal levels of mRNA encoding rMOR-1, rMOR-1A, rMOR-1G1 and rMOR-1R remained invariable in the spinal cord of females as well as males following chronic morphine.

Chronic systemic morphine upregulates density of MOR in the spinal cord of male rats

Radioligand binding studies were employed to determine the effect of chronic morphine on the MOR density and agonist affinity in spinal cord of male rats (Fig. 2). [^{3}H]DAMGO B_{max}±SEM and K_D±SEM in lumbar spinal cord of naïve male rats were 109.5±3.0 fmol/mg protein and 1.49±0.04 nM, respectively. Following chronic systemic morphine there was a

small but significant increase (13%; $p<0.05$) in [^{3}H]DAMGO B_{max} (125.3±4.5 fmol/mg protein) without a significant change in K_D (1.60±0.03 nM).

Discussion

This is the first report to suggest that chronic systemic treatment with morphine, sufficient to produce profound analgesic tolerance and dependence at the time of sacrifice (Villar & Bhargava 1992), not only alters the content of spinal cord mRNAs that encode specific MOR splice variants but also does so in a sex-dependent fashion. Sexually dimorphic alterations in the relative abundance of selective MOR variants, which could have unique signaling attributes, provides a molecular context for understanding sex-based accommodations to chronic morphine that have been reported previously (Chakrabarti et al. 2012).

In spinal cord of males, but not females, chronic morphine was found to augment mRNA encoding rMOR-1C1 and rMOR-1B2. Poor correlation between steady state isoform-specific mRNA and the protein it encodes has been reported (Mons & Cooper 1994). Nevertheless, it seems reasonable to conjecture that a selective increase in the mRNA encoding rMOR-1B2 and rMOR-1C1 likely suggests a parallel change in the content and activity of rMOR-1B2 and rMOR-1C1 receptor protein. This inference is supported by saturation binding analyses, which revealed that a small but significant increase in MOR receptor density accompanies the increment in rMOR-1B2/rMOR-1C1 mRNA following chronic systemic morphine.

Analgesic tolerance formation was not directly assessed in the current study. However, the regimen of chronic systemic morphine exposure is well established to produce profound analgesic tolerance and dependence at the time of animal sacrifice (Villar & Bhargava 1992). Importantly, sexual dimorphism in the magnitude of analgesic tolerance remains controversial in both rats (Craft et al. 1999, Holtman *et al.* 2004) and mice (Hosseini *et al.* 2010, Kest *et al.* 2000). Thus, it is highly unlikely that differential magnitudes of analgesic tolerance in males vs. females per se could explain the observed sex-dependent effects of chronic morphine on spinal rMOR-1B2/rMOR-1C1 mRNA. Moreover, since the magnitude of differences in MOR agonist efficacy and potency among splice variants is relatively small in comparison to the magnitude of tolerance that can develop, it also seems unlikely that presumptive tolerance-associated upregulation of rMOR-1C1 and rMOR-1B2 per se would significantly contribute to tolerance by altering the agonist response profile to mu agonists (Pasternak & Pan 2013).

C-terminal splice variants of MOR have been reported to differ in their endocytotic response to chronic morphine (Koch *et al.* 2001, Koch *et al.* 1998). However, given the well documented disconnects between the ability of opioid agonists to induce MOR endocytosis and/or G protein uncoupling vs. analgesic tolerance (Sternini *et al.* 1996, Madia *et al.* 2012), the differential intracellular trafficking of C-terminal splice variants, per se, is not likely to be a primary factor modulating morphine analgesic tolerance in vivo.

There are, however, unique structural characteristics of rMOR-1C1 and rMOR-1B2, which reside in the composition of the tip of the C-terminus that could influence their functionality. The C-terminal twelve amino acids (aa) of rMOR-1, LENLEAETAPLP, are replaced in rMOR-1C1 by 65 aa, PALAVSVAQIFTGYPSPTHGEKPCKSYRDRPRPCGRTWSLKSRAESNVEHFHCGAA LI-YNNVNFI [encoded for by exon 7 (bp 44966586-4496614), exon 8 (bp 45027017-450027076), exon 9a (bp 45034092-45034145) and exon 9b (bp 45032373-45032473) of the OPRM gene located on chromosome 1]. In rMOR-1B2, the C-terminal twelve aa of rMOR-1 are replaced by seven aa, EPQSVET, (Zimprich *et al.* 1995, Pan 2005, Pan et al. 2005b), encoded by exon 5 [exon 5b (bp 44845374- 44847120) and 5a (bp 44847121-44847400) of the OPRM1 gene]. Importantly, the unique C-terminus of rMOR-1B2/rMOR-1C1 contains numerous consensus sequences for phosphorylation by a variety of kinases that are not present in rMOR-1. (e.g., protein kinase C, protein kinase A, casein kinase II, ribosomal factor S6 kinase, cyclin-dependent kinase 5 and cGMP dependent protein kinase, etc).

Phosphorylation patterns of G protein coupled receptors not only influence the nature of the particular G protein to which receptors couple (Lefkowitz *et al.* 2002, Zamah *et al.* 2002), but also influence biased agonism, agonist-specific stabilization of receptor conformations that preferentially activate specific downstream signaling pathways (Kahsai *et al.* 2011). Moreover, the induction by chronic morphine of newly synthesized MOR variants and the intracellular trafficking of newly synthesized MORs could themselves constitute signals that herald novel adaptations. These considerations suggest that the male-specific upregulation of spinal rMOR-1B2/rMOR-1C1 in response to chronic morphine could substantially influence the aggregate signaling consequences of mu opioids. In this regard, it is relevant to note that a derivative of the endogenous MOR ligand, endomorphin 2, has been shown to elicit analgesic tolerance and related biochemical sequelae (Muranyi *et al.* 2013). Thus, male-specific adaptations to chronic morphine described herein could be relevant to physiological adaptations to stimuli that result in sustained utilization/activation of the endogenous MOR analgesic system. This is particularly so since MOR-1C1 has been shown to be present in laminae I/II of rat and human spinal cord (Abbadie et al. 2000b, Abbadie *et al.* 2000a), areas rich in endomorphin 2 (Martin-Schild *et al.* 1999, Schreff *et al.* 1998).

Recently, we reported on the heterodimerization of MOR with KOR in spinal cord that was not only sex-dependent but also dependent on the stage of the estrus cycle (Chakrabarti et al. 2010, Liu et al. 2011), the bases for which remain unknown. We had speculated that sex and estrus cycle dependence of MOR/KOR heterodimerization resulted from sex- and estrus cycle-dependent expression of spinal MOR variants. However, this is unlikely in light of the current finding that spinal expression levels of mRNA encoding each of the six splice variants studied did not vary between diestrus and proestrus females.

Surprisingly, spinal levels of mRNA encoding rMOR-1B2 and rMOR-1G1 were significantly higher in the spinal cord of diestrus female vs. male rats. The physiological/pharmacological significance of this difference remains to be defined. However, it should be noted that differences in analgesic responsiveness among diestrus, proestrus and male rats have been reported (Boyer et al. 1998). Furthermore, since diestrus is the longest single

stage of the estrus cycle, studies comparing males and (non-cycled) females most likely involve females in diestrus. Therefore, differences in spinal rMOR-1B2/rMOR-1G1 could undergird, at least in part, male-female differences in opioid antinociception that have been reported, definitive proof of which will require the development of MOR variant-selective agonists and antagonists.

In summary, the sexually dimorphic ability of chronic systemic morphine to upregulate spinal rMOR-1C1 and rMOR-1B2 mRNA could suggest that spinal MOR variants are differentially recruited in males vs. females to mediate sex-dependent adaptations to chronic morphine, e.g., (Chakrabarti et al. 2012). Knowledge of unique signaling properties of rMOR-1C1 and rMOR-1B2, which reside in the composition of the tip of the C-terminus, could point the way to defining the molecular determinants of sex-dependent tolerance and withdrawal mechanisms and treatments thereof.

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Abbreviations

MOR	Mu-opioid receptor
aa	amino acids
G6PD	Glucose-6-phosphate dehydrogenase

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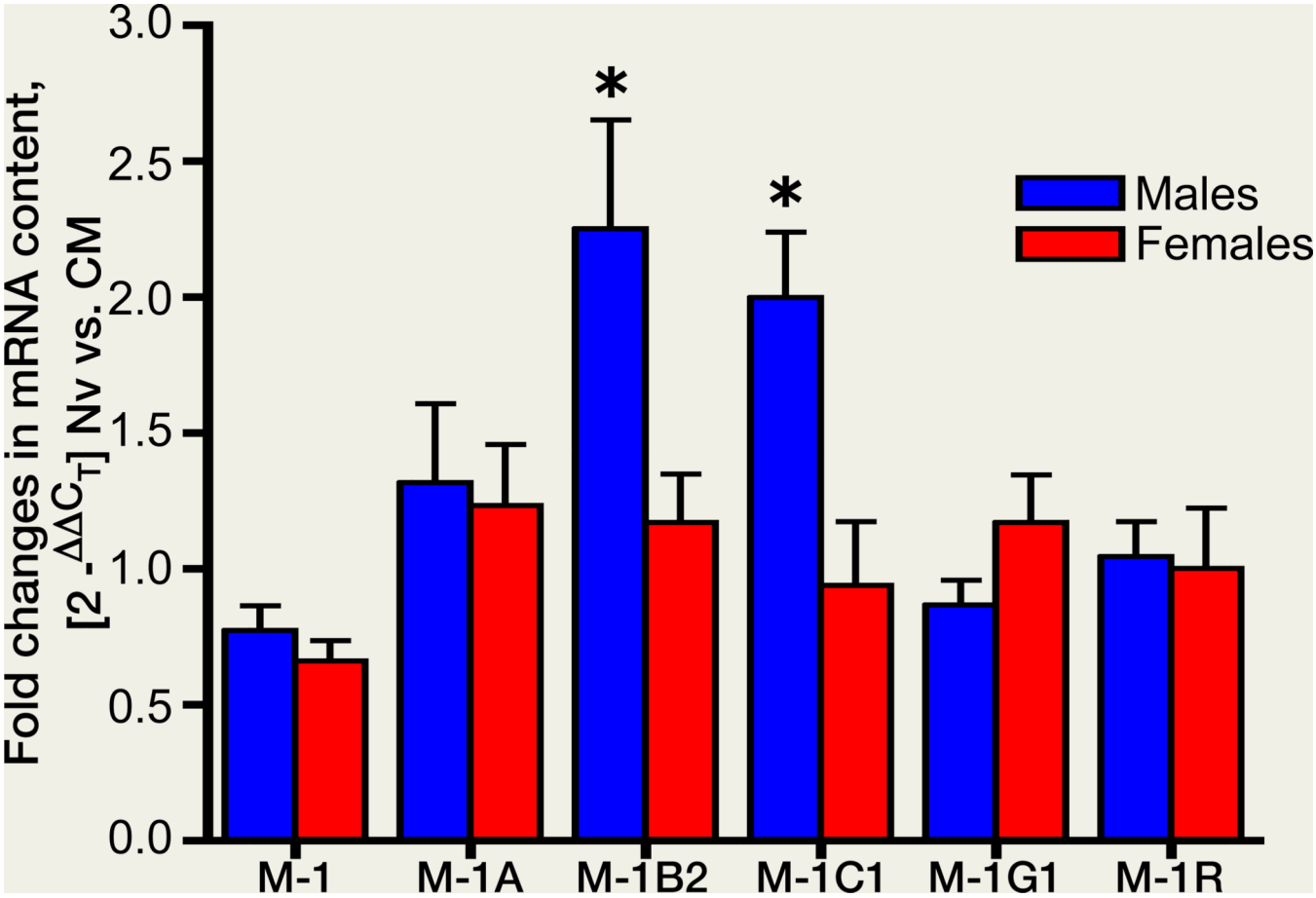

Figure 1. Sex-dependent effect of chronic morphine on the mRNA expression of MOR variants

Male and female rats were treated with escalating doses of morphine base pellets (see methods). Data are calculated as fold change in MOR mRNA content, where $2^{-\Delta\Delta CT}$ represents the change in spinal mRNA content in opioid naïve (Nv) vs. chronic morphine treated (CM) groups relative to that of rMOR-1R. Chronic morphine significantly upregulated two splice variants of MOR, rMOR-1B2 (M-1B2) and rMOR-1C1 (M-1C1) in only males (*= ~ 2 fold; $p<0.05$). n=5-8).

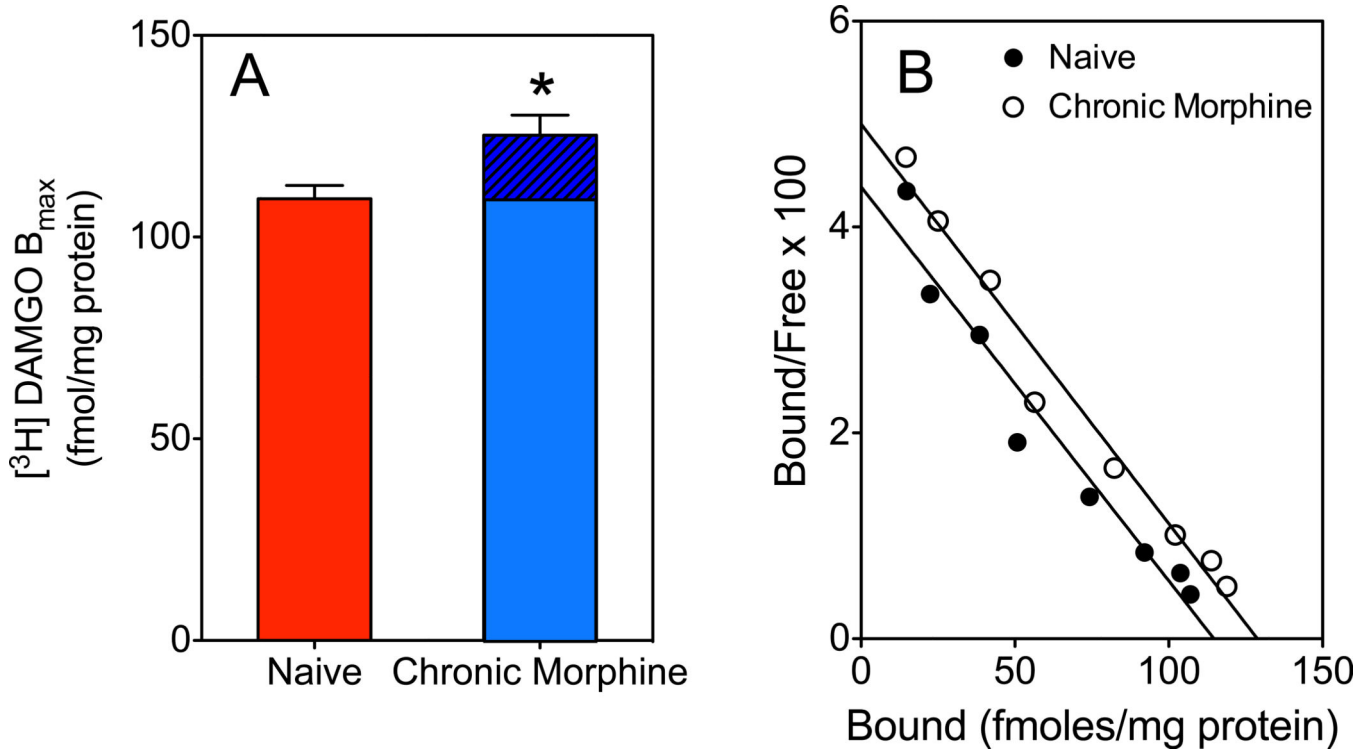

Figure 2. Effect of chronic systemic morphine on MOR density in spinal cord of male rat

Lumbar spinal tissue obtained from two placebo or chronic morphine-treated male rats were dissected and pooled for each saturation binding experiment using [^{3}H]DAMGO. (A) Mean [^{3}H]DAMGO B_{max}±SEM was determined in spinal cord from naïve (109.5±3.0 fmol/mg protein) and chronic morphine treated (125.3±4.5 fmol/mg protein) groups (*$p<0.05$; n=6). Hatched portion indicates the increment in [^{3}H]DAMGO B_{max} following chronic-morphine treatment. (B) A representative scatchard plot is shown for naïve and chronic morphine-treated spinal cord. No significant difference was observed between [^{3}H]DAMGO K_Ds in naïve (1.49±0.04 nM) and morphine-treated spinal cord (1.60±0.03 nM) group ($p>0.05$).

Table 1

Relative spinal content of mRNA encoding MOR variants in diestrus, proestrus female and male rats

MOR Variants	Relative mRNA levels ($2^{-\Delta C_T}$)		
	Diestrus Female	Proestrus Female	Male
rMOR-1	1.671 ± 0.222	1.251 ± 0.255	1.591 ± 0.205
rMOR-1A	0.3171 ± 0.06	0.2498 ± 0.054	0.14912 ± 0.0258
rMOR-1B2	0.00573 ± 0.001*	0.00315 ± 0.0006	0.00177 ± 0.0002
rMOR-1C1	0.000563 ± 0.0001	0.000772 ± 0.0003	0.000393 ± 0.00003
rMOR-1G1	0.0821 ± 0.0194*	0.03489 ± 0.015	0.02498 ± 0.0038
rMOR-1R	0.000617 ± 0.0001	0.000415 ± 0.00034	0.000136 ± 0.0001

The C_T values, number of amplification cycles to reach threshold detection, were obtained for MOR variants and for the control gene G6PD using RT-PCR. Comparative mRNA levels of each MOR variant were calculated as $2^{-\Delta CT}$ where ΔC_T is the difference between the C_T for each MOR variant and that of G6PD (n=4-8).

* $p<0.05$ in comparison to males.