HHS Public Access

Author manuscript

J Neuroimmune Pharmacol. Author manuscript; available in PMC 2023 June 01.

Published in final edited form as:

J Neuroimmune Pharmacol. 2022 June ; 17(1-2): 277–288. doi:10.1007/s11481-021-10009-4.

Modulation of OPRM1 alternative splicing by morphine and HIV-1 Nef

Martina Donadoni[1], **Wenfei Huang**[2], **Shadan S. Yarandi**[1], **Tricia H. Burdo**[1], **Sulie L. Chang**[2,*], **Ilker K. Sariyer**[1,*]

[1]Department of Neuroscience and Center for Neurovirology, Temple University Lewis Katz School of Medicine, Philadelphia, PA 19140

[2]Institute of NeuroImmune Pharmacology and Department of Biological Sciences, Seton Hall University, South Orange, NJ. USA.

Abstract

Clinically used opioids, such as morphine, activate the mu opioid receptor (MOR) encoded by Opioid Receptor Mu 1 (OPRM1) gene. Examination of the opioid receptor genes showed that the human OPRM1 pre-mRNA undergoes extensive alternative splicing events and capable of expressing 21 isoforms. However, characterization of OPRM1 signaling is generalized, and only one isoform (MOR-1) has been extensively studied. Compounding this issue is the increasing significance of intravenous drug abuse in HIV neuropathogenesis. Here, we investigated the molecular impact of morphine and HIV-1 on regulation of OPRM1 pre-mRNA slicing in *in vitro* and *in vivo* models. Our results suggested that morphine treatment specifically induces the alternative splicing of MOR-1X isoform among the other isoforms analyzed in neuronal cells. Interestingly, alternative splicing and expression of MOR-1X isoform was also induced in postmortem brain tissues obtained from people with HIV (PWH). Additionally, treatment of control rats with morphine induced alternative splicing of MOR-1X in the brain regions involved in the reward pathways. More interestingly, HIV-1 transgenic (HIV-1Tg) rats, showed an additive induction of MOR-1X isoform with the exposure to morphine. To further assess the possible role of HIV secretory proteins in alternative splicing of OPRM1 gene, we analyzed the impact of HIV-1 Tat, gp120 and Nef proteins on alternative splicing of MOR-1X isoform. While the Tat and gp120 had no visible effects, treatment of neurons with Nef induced MOR-1X alternative splicing that was comparable to treatment with morphine. Altogether, our results suggest that HIV-1 may

*Corresponding Authors: Ilker K. Sariyer, D.V.M., Ph.D., Department of Neuroscience and Center for Neurovirology, Lewis Katz School of Medicine at Temple University, 3500 North Broad Street, Medical Education and Research Building Room 753, 7th Floor, Philadelphia, PA 19140, isariyer@temple.edu, Sulie L. Chang, Ph.D., Institute of NeuroImmune Pharmacology and Department of Biological Sciences, Seton Hall University, South Orange, NJ. USA. sulie.chang@shu.edu.

Authors' contributions

Conceived and designed the experiments: IKS and SLC. Performed the experiments: MD, WH, SSY. Analyzed the data: MD, WH, IKS and SLC. Contributed reagents/materials/analysis tools: IKS, THB, SLC. Wrote the paper: IKS, SLC, and MD.

Conflicts of interest/Competing interests

The authors declare no conflict of interest.

Ethics Approval

Human postmortem brain samples were obtained and used in accordance with Temple University Human Subjects Protections, with the approval of the institutional review board. The experimental protocol using HIV-1Tg and F344 rats was approved by the Institutional Animal Care and Use Committee (IACUC) at Seton Hall University, South Orange, NJ.

alter MOR isoform expression with Nef protein by amplifying the rate of MOR-1X alternative splicing induced by morphine.

Keywords

HIV; Nef; opioids; OPRM1; MOR-1; MOR-1X; alternative splicing; dependence

INTRODUCTION

Abuse of addictive substances including morphine has been identified as a key comorbidity of human immunodeficiency virus (HIV) infection (Reddy et al. 2012; Tiwari et al. 2013; Chang et al. 2014). In addition, there is a high prevalence of prescription opioid use among people with HIV (PWH), with many individuals having prescribing patterns associated with a high risk of dependence. Approximately 40% of PWH receive an opioid prescription, with high-risk use occurring in a third of these individuals. Many studies have found that the use and abuse of addictive substances such as opioids, accelerates the progression of HIV-1 infection and HIV-associated neurocognitive dysfunction (Tiwari et al. 2013; Kennedy and Zerbo 2014; Dahal et al. 2015). Advances in combination anti-retroviral therapy (cART) in the last 25 years have been successful in limiting the HIV viral load and maintaining a relatively healthy immune response, allowing the life expectancy of PWH to approach that of the general population (High et al. 2012). However, even with cART, HIV-1 viral proteins are still expressed and eradication of the virus, particularly in the brain, does not occur (Saylor et al. 2016). Currently, the clinical challenge in the treatment of HIV infection is inflammation of the central nervous system (CNS) and its subsequent neurological disorders. Studies using rodent models and human subjects have shown that HIV infection causes alterations in the structure and function of the CNS reward pathways and increases the likelihood that PWH will become addicted to one or more of these substances. Clinical evidence shows that PWH are more likely to abuse addictive substances including morphine, heroin, alcohol, nicotine, and methamphetamine than those in the general population (Molitor et al. 1998; Nath et al. 2002; Avdoshina et al. 2010). According to the UNODC World Drug Report, approximately 11 million people inject drugs and, among them, about 1.4 million are living with HIV worldwide (UNODC World Drug Report, 2020). The percentage of PWH who use morphine/heroin is 6–7 fold greater than that in the general population in the United States. HIV is most likely involved in increasing the rate of substance abuse including morphine in PWH; however, host and viral mechanisms underlying relationship between HIV infection and morphine use disorders remains to be elucidated. Multiple studies in mice, rats, and humans have shown that OPRM1 pre-mRNA undergoes extensive splicing to give multiple isoforms (Bare et al. 1994; Kvam et al. 2004). The human *Oprm1* gene consists of 11 exons and encodes for 21 alternatively-spliced isoforms (Shabalina et al. 2009). There are several types of alternative splicing events associated with MOR pre-mRNA splicing. The first type is exon skipping, in which an exon is spliced out of the mature mRNA transcript together with its flanking introns. The second and third types involve the selection of alternative 3′ and/or 5’ splice sites. The fourth type is intron retention, in which an intron region remains in the mature mRNA transcript. Less frequently, complex events that give rise to alternative

transcripts include mutually exclusive exons (Kim et al. 2008; Hui 2009; Keren et al. 2010). Several signaling pathways have been implicated in the regulation of alternative splicing events of OPRM1. These pathways include Ras/MEK/ERK, Rac/JNK/p38 MAPK, Ras/PI3-kinase/AKT and Ca2+ /calmodulin/CaMK IV (Tarn 2007). Studies examining alternative splicing of the OPRM1 have found differences in ligand binding affinity among these receptors (Pan et al. 2003, 2005; Pasternak et al. 2004; Oldfield et al. 2008; Ravindranathan et al. 2009; Pasternak 2010). Furthermore, these receptors seem to have different rates of GTP binding, internalization, and re-sensitization in response to different μ-selective ligands (Koch et al. 1998, 2001; Abbadie and Pasternak 2001; Mizoguchi et al. 2003; Tanowitz et al. 2008). We previously studied the effect of morphine exposure on alternative splicing of MOR-1 and MOR-1X isoforms and found that morphine preferentially induced the alternative splicing of MOR-1X isoform over MOR-1 (Regan et al. 2016). Here, we further investigated the possible involvement of HIV in alteration of pre-mRNA splicing of OPRM1 induced by morphine by using *in vitro* and *in vivo* models.

MATERIALS AND METHODS

Postmortem brain tissues from PWH

Autopsy brain tissues were obtained from National NeuroAIDS Tissue Consortium (NNTC) from 8 control and 8 HIV+ subjects. Subjects were male with median age of 46.3 years. Three HIV+ brains were from cognitively impaired (3 had HAND diagnoses, of which two had MND and one subject had possible HAD). Four specimens came from people on ART. The median CD4 count was low with 48 T cells /uL of blood (IQR, 13–21). Cortex tissues were processed for total RNA isolation by using a commercial tissue RNA extraction kit (New England Biolabs).

Cell culture and reagents

SH-SY5Y cells, a human neuroblastoma cell line, was obtained from the American Type Culture Collection (ATCC) and was cultured and maintained according to ATCC's instructions. Human primary neurons, human primary astrocytes, and rat primary neurons were provided by the Comprehensive NeuroAIDS Center (CNAC) tissue culture core at Lewis Katz School of Medicine (LKSOM) Temple University. The primary cells under CNAC core are isolated and cultured in compliance with ethical guidelines of the National Institutes of Health and LKSOM. Primary rat and human neurons were provided in six-well tissue culture dishes coated with laminin and Poly-L-ornitine. Neurons were cultured and maintained as described before (Donadoni et al. 2019) in neurobasal medium (Gibco) containing a B-27 supplement (Gibco/Thermo Fisher Inc., Waltham, MA, USA), 0.05% GlutaMAX (Gibco) and gentamicin (10 μg/ml) at 37°C under 5% atmospheric CO_2. Primary human (PHFA) were obtained from the fetal brain of donors provided by the CNAC core and cultured as described before (Craigie et al. 2018) in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin/streptomycin (100 μg/ml), GlutaMax (100 μg/mL) and insulin (100 μg/mL). Cultured PHFA were maintained at 37° C in a humidified atmosphere with 5% CO_2. Recombinant Tat101 and gp120 were obtained from Abcam (#ab83358 and #ab73769, respectively).

Semi-quantitative RT-PCR analysis of OPRM1 alternative splicing

SH-SY5Y cells, primary human neurons, or primary rat neurons were exposed to various concentrations of morphine (0.1, 1.0, 5.0 μM) based on experimental designs. Cells were harvested at various time points, and total RNA was extracted with an RNA extraction kit (New England Biolabs) according to the manufacturer's instructions. Total RNA samples were also extracted from postmortem brain tissues from PWH and various brain regions of F344 control and HIV-1-Tg rats. RT-PCR reactions were performed as described previously (Donadoni et al. 2019). Briefly, RNA samples were first treated with DNase I followed by phenol/chloroform extraction and ethanol precipitation. cDNAs were synthesized by using M-MuLV reverse transcriptase and RNA templates were removed from reactions by RNase H digestion. A total of 100 ng cDNA was used as template for PCR reactions. Alternatively, spliced isoforms of OPRM1 gene were amplified by PCR using primers listed on Table 1. β-Actin and GAPDH mRNA were also amplified from the same set of RNA samples by RT-PCR as internal controls. Amplified gene products were resolved on 1– 1.5% DNA-agarose gels and visualized by ethidium bromide staining.

Animals

Both male and female HIV-1Tg rats (12 in total) and the control F344 rats (12 in total) at 3–4 wks of age were purchased from University of Maryland (Baltimore, MD). At 8 weeks of age, each HIV-1Tg rats or control F344 rats were given subcutaneous (s.c.) implementation of two pellets of morphine (75 mg free base) or placebo on Day 1 and four pellets on Day 2. The animals were anesthetized lightly with methoxyflurane during pelleting procedure. On Day 5, animals were sacrificed to collect the brain and dissect various brain regions. RNA samples were isolated from prefrontal cortex (PFC), hippocampus (HIPP) and striatum (STR) for semi-quantitative RT-PCR analysis of OPRM1 isoforms. The experimental protocol using HIV-1Tg and F344 rats was approved by the Institutional Animal Care and Use Committee (IACUC) at Seton Hall University, South Orange, NJ.

Purification and characterization of control and Nef-EVs from culture media

Purification and characterization of extracellular vesicles (EVs) and Nef-EVs from glial cells were recently described by us (Sami Saribas et al. 2017; Vakili et al. 2020). Briefly, primary astrocytes were seeded in 100 mm dishes, transduced with Adeno-Null and Adeno-Nef at MOI of one. Adeno-Null and Adeno-Nef constructs were generated and reported previously (Sami Saribas et al. 2017). The cultured media was collected at 72 hours post-transductions. Approximately 10mL growth media from cells was first centrifuged at 3000× *g* for 30 min at 4 °C (Eppendorf Centrifuge, 5804R) to clear cell debris followed by a centrifugation at 10,000× *g* for 30 min at 4 °C. EVs were purified from supernatants utilizing commercial ExoQuick solution (System Biosciences, Palo Alto, CA) according to manufacturer's instructions. EVs from control and Nef expressing cells were analyzed for their size and concentration by ZetaView instrument and Software 8.02.31 (Cambridge, UK) with specific analysis parameters: maximum particle size: 1000, minimum particle size 5, and minimum particle brightness: 20.

RESULTS

Morphine induces alternative splicing of MOR-1X isoform in neuronal cells

OPRM1 pre-mRNA undergoes extensive alternative splicing events with a potential of 21 known isoform expression in humans. Fig.1A schematize the exon/intron structure of OPRM1 pre-mRNA and alternative splicing patterns of MOR-1, MOR-1X, MOR-1A, and MOR-1i. In order to gain insight into the morphine effects on OPRM1 isoforms, SH-SY5Y cells, a model for human dopaminergic neurons, were treated with 0.1, 1.0, and 5.0 μM morphine for 6 hrs. Total RNA was extracted from the cells and analyzed by semi-quantitative RT-PCR for several MOR isoforms including MOR-1, MOR-1A, MOR-1X, MOR-1i, MOR-1A2, MOR-1B1, MOR-1B2, MOR-1B3, MOR-1B4, MOR-1B5, and MOR-1O. Interestingly, in the absence and presence of morphine treatments, RT-PCR bands corresponding to the expression of MOR-1A2, MOR-1B1, MOR-1B2, MOR-1B3, MOR-1B4, MOR-1B5, and MOR-1O were not observed (data not shown); suggesting that those isoforms neither expressed by SH-SY5Y cells nor induced by morphine treatments. On the other hand, expressions of MOR-1, MOR-1A, MOR-1X, and MOR-1i isoforms were detected in untreated SH-SY5Y cells (Fig. 1B, lane 1). More interestingly, among these isoforms, only MOR-1X isoform was induced in a dose dependent manner in response to morphine treatment (Fig. 1B, lanes 2–4, and Fig. 1C), suggesting that morphine treatment exclusively induced the expression of MOR-1X which possesses an extended intracellular C-termini domain compared to the MOR-1 isoform (Fig. 1D).

Expression of MOR-1X isoform is induced in postmortem brain tissues obtained from PWH

To gain insight into whether OPRM1 alternative splicing is associated with HIV-1 infection in the brain, we analyzed the expression of MOR-1 and MOR-1X isoforms in postmortem brain tissues obtained from PWH. Brains from age matched control (non HIV, n=8) and HIV-1+ (n=8) specimens were analyzed. Total RNA was extracted from tissues and analyzed by RT-PCR for MOR-1 and MOR-1X isoforms. As shown in the upper panels, MOR-1 isoform was robustly expressed in all the tissues with no significant variation in HIV+ vs control brain tissues (Fig. 2A and B, upper panels, the quantification for MOR-1 isoform was not shown). Interestingly, detectible but significantly less MOR-1X expression was also detected in non-HIV control tissues (Fig. 2A, and B, mid panels). More interestingly, MOR-1X levels were significantly increased in all the HIV+ brain samples analyzed (Fig. 2A–B, and C, normalized MOR-1X band intensities were determined by Image J and shown as bar graph). GAPDH mRNA levels were comparable between control and HIV+ brain tissues. These results, for the first time, revealed that MOR-1X isoform is induced in the brains of PWH.

Alternative splicing of MOR-1X is induced in the brain regions of F344 (control) rats treated with morphine and HIV-1Tg rats with an additive induction with morphine exposure

The persistence of HIV viral proteins in HIV-1Tg rat models correlates with upregulation of MOR expression (Chang and Vigorito 2006) in line with enhancement of sensitivity of morphine's anti-nociception as measured by the rat's tail-flick latency (Chang and Vigorito 2006; Chang and Connaghan 2012; Vigorito et al. 2015) and morphine-induced conditioned place preference (CPP) (Homji et al. 2012). As shown above, our data suggest that

MOR-1X isoform is induced in brains of PWH. Therefore, we sought to further investigate the possible induction of MOR-1X isoform in HIV-1Tg rats with and without morphine treatment. In order to determine if morphine and HIV-1 had any impact on alternative splicing of OPRM1 *in vivo*, both male and female HIV-1Tg and F344 control rats were implemented with two pellets of morphine (n=6, 75 mg free base) or placebo (n=6) on Day 1 and four pellets on day 2. On day 5, the animals were sacrificed to collect the prefrontal cortex (PFC), hippocampus (HIPP) and striatum (STR) for total RNA isolation. Total RNA samples were processed for RT-PCR using specific primer pairs for the amplification of MOR-1 and MOR-1X isoforms (Fig. 3A and B). Semi-quantitative analysis of MOR-1 and MOR-1X was performed and shown as bar graph (Fig. 3C and D). Interestingly, MOR-1X isoform was significantly induced in HIV-1Tg rats compared to control F344 in PFC, HIPP, and STR. Moreover, morphine exposure in F344 rats also induced MOR-1X isoform in HIPP and STR of male and only in HIPP of female rats. More interestingly, morphine in HIV-Tg rats showed a synergistic induction of MOR-1X isoform in HIPP and STR regions of both male and female rats. These results revealed a novel molecular interaction between morphine and HIV-1 leading to synergistic induction of MOR-1X isoform.

Effects of HIV-1 Tat, gp120, and Nef on alternative splicing of MOR-1X isoform in primary neurons

As shown in Fig. 2, our results suggested that splicing of OPRM1 was altered and MOR-1X expression was induced in autopsy brain specimens from PWH. More interestingly, our results also indicated that alternative splicing of MOR-1X was induced in the brain regions involved in the reward pathways of F344 rats treated with morphine and HIV-1Tg rats with an additive induction with morphine exposure (Fig. 3). Those results suggested that HIV-1 may contribute to alteration of OPRM1 pre-mRNA splicing induced by morphine. In order to determine possible roles of HIV secretory proteins in alternative splicing of MOR-1X, primary human neurons were treated with recombinant Tat-101 (100 ng/ml), recombinant gp120 (500 ng/ml), and Nef-EVs (1000 EV particles / cell) in the absence of morphine. Recombinant Tat and gp120 were obtained commercially. Nef EVs were purified from culture media of primary human astrocytes transduced with an adenovirus construct expressing Nef. Cells were also treated with morphine (1 μM) and control EVs (1000 EVs/cell). Control and Nef-EVs were characterized by ZetaView nanoparticle tracking instrument for concentration and size distribution (data not shown). Total RNA was extracted from the cells at 8 hrs post-treatments and analyzed by semi-quantitative RT-PCR for MOR-1 and MOR-1X isoforms. Once again, MOR-1X isoform was induced by morphine in primary neurons (Fig. 4A, lane 3). Interestingly, while recombinant Tat (lane 4) and gp120 (lane 7) had no visible effects, treatment of neurons with Nef-EVs (lane 5) resulted in a comparable induction in MOR-1X alternative splicing as morphine caused (Fig. 4B). There was no alteration in MOR-1X expression in cells treated with control EVs.

Nef-EVs and morphine synergistically induce alternative splicing of MOR-1X isoform in primary neurons

In order to further determine combined effects of morphine and Nef-EVs on alternative splicing of MOR-1X isoform and determine if MOR-1X is also expressed/induced in rat neurons, primary human and rat neurons were exposed to either control-EVs or Nef-EVs

(500 EVs per cell) in the presence and absence of morphine treatment (1 μM). Total RNA was extracted from the cells at 24 hrs post-treatments and analyzed by semi-quantitative RT-PCR for MOR-1 and MOR-1X isoforms. MOR-1 was the main isoform expressed by human and rat neurons (Fig. 5A and C). Interestingly, while control-EVs had no effects, treatment of neurons with Nef-EVs or morphine individually was sufficient to induce the MOR-1X in human as well as in rat neurons. More interestingly, combined treatment of human and rat neurons with Nef-EVs and morphine resulted in a greater induction of MOR-1X isoform. Semi-quantitative analysis of MOR-1 and MOR-1X were performed and shown as bar graph (panels B and D). These results suggest that HIV-1 Nef protein and morphine may synergistically induce MOR-1X alternative splicing in primary human and rat neurons.

DISCUSSION

Limited number of studies suggest that HIV-1 infection may alter the pre-mRNA splicing of OPRM1 (Dever et al. 2012, 2014). The studies primarily focused on few MOR isoforms in control and HIV-1 infected astrocytes and found that MOR-1X and MOR-1A isoforms were significantly induced in infected astrocytes (Dever et al. 2012). We have reported that morphine induced the exclusive expression of MOR-1X isoform and upregulated the expression of splicing regulatory protein ASF/SF2 in neuronal cells (Regan et al. 2016). Our results from the current study suggested that alternative splicing and expression of MOR-1X isoform was also induced in postmortem brain tissues obtained from PWH. We also have reported that glial cells infected with HIV-1 released Nef protein in association with extracellular vesicles (Nef-EVs), which were readily taken up by neurons (Saribas et al. 2017). In an effort to determine possible role of HIV secretory proteins in alternative splicing of MOR-1X, our results suggested that, while recombinant Tat and gp120 had no effects, treatment of neurons with Nef-EVs caused a comparable induction in MOR-1X alternative splicing with morphine. More interestingly, Nef-EVs with morphine synergistically induced alternative splicing of MOR-1X isoform in human and rat neurons. Additionally, alternative splicing of MOR-1X was induced in the brain regions involved in the reward pathways of F344 control rats treated with morphine and HIV-1Tg rats with an additive induction with morphine exposure.

Since the advent of cART, PWH are expected to have a close to a normal life expectancy, and there has been a shift in HIV research from studying the detrimental impacts of addictive substances including opioids on PWH to investigation of whether HIV infection itself can impact dependency to addictive substances. In this extend, based on our current and previous observations, we have developed a model for the possible impact of HIV on OPRM1 signaling and its role in alternative splicing and expression of MOR isoforms (Fig. 6). Opioids including morphine trigger intracellular events through traditional MOR-1 receptor leading to modulation of splicing regulatory proteins including ASF/SF2. In order to determine possible roles of HIV secretory proteins in alternative splicing of MOR-1X, we treated primary human neurons with recombinant Tat-101, recombinant gp120, and Nef-EVs. HIV proteins Tat and gp120 are actively secreted from infected astrocytes, microglia, and macrophages, and can be rapidly internalized by a variety of cell types, including neurons (Brew et al. 1995; Saha and Pahan 2003; Jana and Pahan 2004; Gurwitz et al. 2017).

Since neurons are not prone to the HIV infection, in order to mimic *in vivo* conditions, we treated neurons with recombinant Tat and gp120 proteins with dosages reported in the literature for this type of studies (Bardi et al. 2006; Gibellini et al. 2011; Mohseni Ahooyi et al. 2018; Natarajaseenivasan et al. 2018). On the other hand, unlike Tat and gp120, we and others showed that Nef is released from infected cells in association with EVs (Sami Saribas et al. 2017; McNamara et al. 2018; Yarandi et al. 2021). In the HIV brain, Nef protein is expressed and released in association with extracellular vesicles by latently infected cells including microglia and astrocytes (Ranki et al. 1995; Raymond et al. 2011; McNamara et al. 2018). Nef-EVs are shown to be taken up by neurons (Sami Saribas et al. 2017) and impact cellular and molecular functions of the cells (Khan et al. 2016; Raymond et al. 2016). Based on our knowledge, there is no free Nef protein rather than the EV associated form exists in the extracellular matrix. Therefore, in our preliminary experiments, Nef EVs were purified from culture media of primary glial cells transduced with an adenovirus construct expressing Nef protein and used for the treatment of neurons. In addition to the additive effect observed in combination with morphine, Nef-EVs were also capable of inducing alternative splicing of MOR-1X isoform in the absence of morphine in neurons. Morphine effects on MOR-1X isoform was linked to the induction of ASF/SF2 expression levels (Regan et al. 2016). However, whether or not Nef also uses the same mechanism as morphine uses still remains to be elucidated.

Given the importance of the C-terminal tail in G-protein coupled receptor (GPCR) signaling, OPRM1 isoforms that have an alternative C-terminal tail, such as MOR-1X, may have significant functional consequences, particularly in opioid dependence. In the MOR-1X mRNA transcript, the mutually exclusive exon X is incorporated into the mature mRNA transcript following exon 3. This results in a substantially longer, C-terminal tail that is unique to the MOR-1X isoform (Fig. 6). All of the known C-terminal and N-terminal MOR isoforms identified have distinct regional, cellular, and subcellular distributions (Abbadie et al. 2000, 2001, 2002, 2004; Ständer et al. 2002; Zhang et al. 2006; Pasternak 2010), and possible functional differences. We previously showed significant differences in MAPK signaling in cells expressing MOR-1 and MOR-1X isoforms, specifically in the ERK RSK1 signaling (Regan et al. 2016). Supported with these observations, it is possible that MOR-1X isoform may signal in a unique way that may lead to the induction of key genes involved in opioid dependence phenotype. However, the exact mechanism of MOR-1X signaling and whether it has any association with the expression of genes involved in opioid dependence remains to be determined.

Altogether, our results provide empirical evidence to demonstrate synergistic involvement of HIV-1 and morphine in altered pre-mRNA splicing of the μ-opioid receptor leading to preferential expression of MOR-1X isoform with molecular and functional implications. In addition, it opens a new avenue of research for further understanding of the role of HIV-1 in enhanced opioid dependence seen in PWH.

Acknowledgments

Authors wants to thank to present and past members of the Neuroscience Department and Center for Neurovirology at Lewis Katz School of Medicine (LKSOM) for sharing of ideas and reagents.

Funding

This work was supported, in part, by grants (DA07058, DA043448, AA025398) awarded by the NIH to IKS, SLC, and THB. SSY was supported by Interdisciplinary and Translational Research Training in NeuroAIDS (T32MH079785). The study utilized services offered by core facilities of the Comprehensive NeuroAIDS Center (CNAC NIMH Grant Number P30MH092177) at LKSOM at Temple University. This publication was made possible from NIH funding through the NIMH and NINDS Institutes by the following grants: Texas NeuroAIDS Research Center: U24MH100930, California NeuroAIDS Tissue Network: U24MH100928, National Neurological AIDS Bank: U24MH100929, Manhattan HIV Brain Bank: U24MH100931, Data Coordinating Center: U24MH100925. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of the NNTC or NIH.

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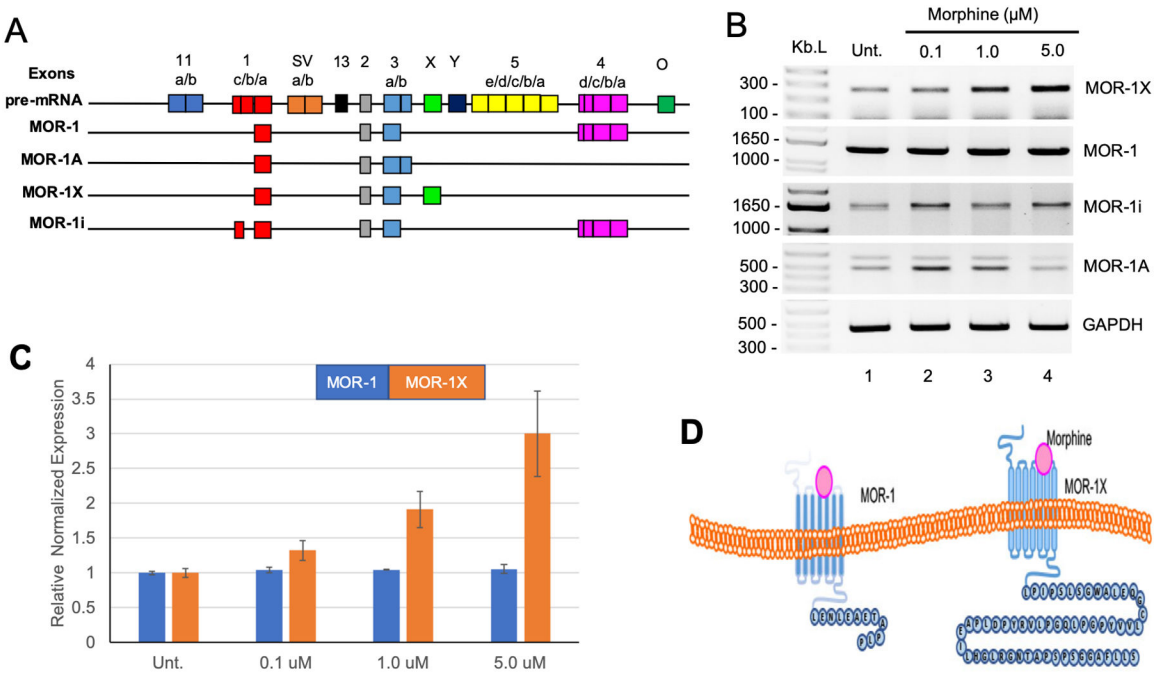

Figure 1: Morphine selectively induces alternative splicing of MOR-1X isoform in SH-SY5Y cells.

A. Splicing patterns of selected OPRM1 isoforms are illustrated according to a comparative analysis of exon and intron inclusions. Top schematic represents positions of known exonic regions within the human OPRM1 pre-mRNA. B. SH-SY5Y cells were treated with morphine (0.1, 1.0, and 5.0 uM) for 6hrs. Total RNA samples were processed by RT-PCR utilizing unique primers for each isoform. GAPDH was also amplified from same samples as internal control. C. Normalized MOR-1 and MOR-1X band intensities from panel B were determined by Image J software and shown as relative expression in bar graph. D. Schematic illustration and comparison of C-term amino acid sequences of MOR-1 and MOR-1X isoforms.

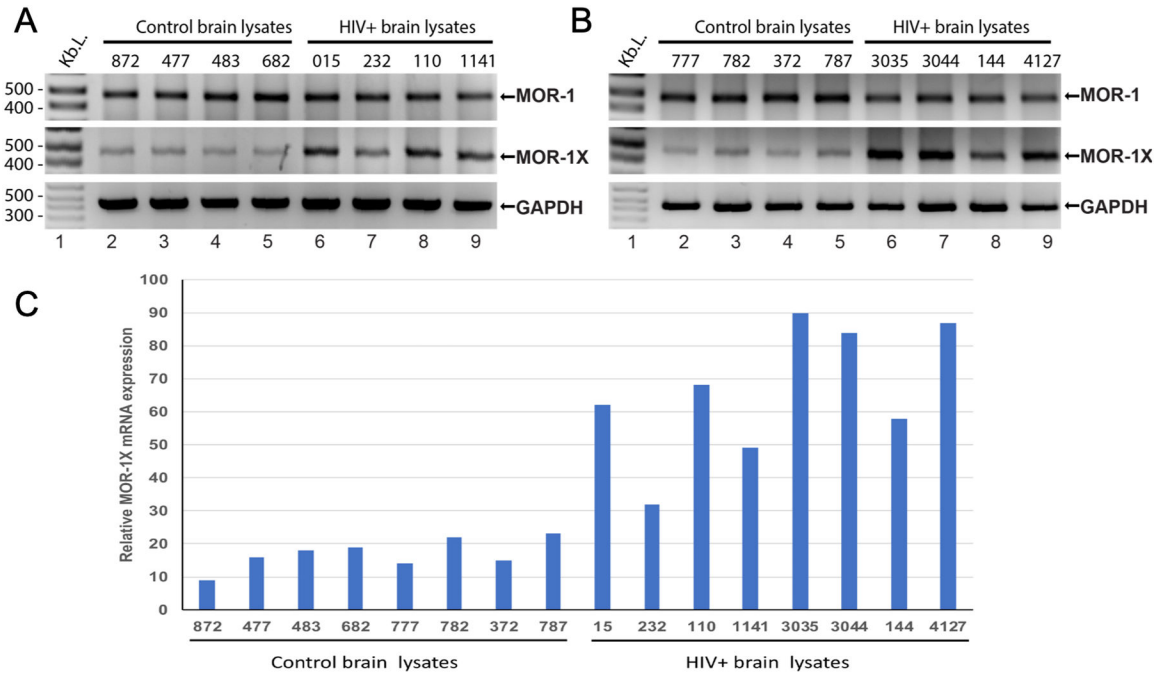

Figure 2: Expression of MOR-1 and MOR-1X isoforms in postmortem brain tissues obtained from PWH:

A-B: Postmortem human brain tissues from control (non HIV, n=8) and HIV-1+ (n=8) were processed for RNA extraction and RT-PCR analysis for MOR-1 and MOR-1X isoforms. GAPDH was also amplified in same samples and shown as internal control. C. MOR-1X band intensities normalized to GAPDH from the panels A and B were determined by Image J and shown as bar graph.

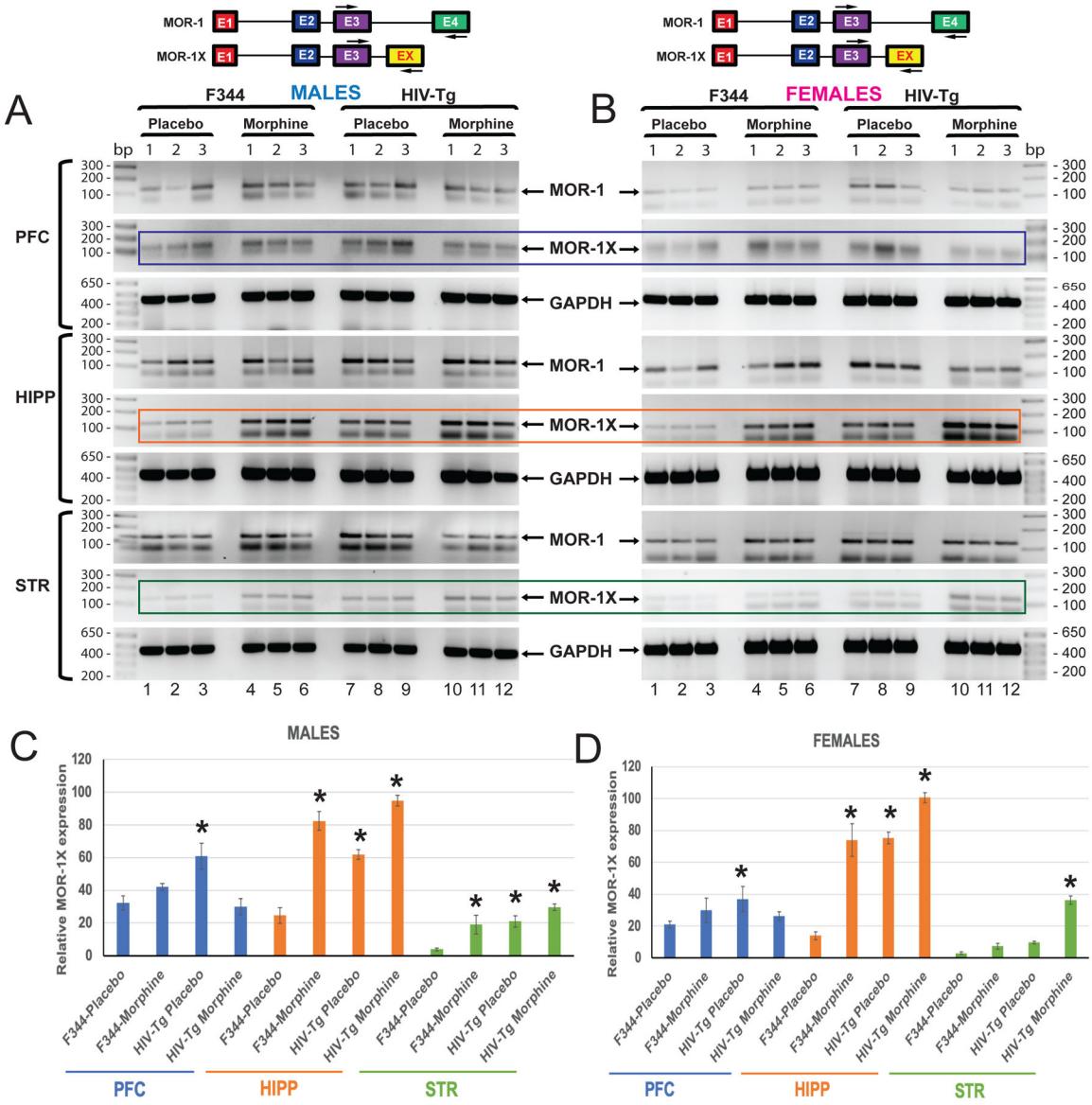

Figure 3: Expression of MOR-1 and MOR-1X isoforms in brain regions of F344 rats and HIV-1Tg rats treated with morphine.

Both male (A) and female (B) HIV-1Tg and F344 control rats were implemented with two pellets of morphine (n=3, 75 mg free base) or placebo (n=3) on Day 1 and four pellets on day 2. On day 5, the animals were sacrificed to collect the prefrontal cortex (PFC), hippocampus (HIPP) and striatum (STR) for total RNA isolation. Total RNA samples were processed for RT-PCR for the amplification of MOR-1 and MOR-1X isoforms. GAPDH was also amplified in same samples. C. Semi-quantitative analysis of MOR-1 and MOR-1X in panels A (males) and B (females) were performed using the Image J software to determine normalized band intensities and shown as bar graph. (n=3 per group, mean, SEM; $P<0.05$).

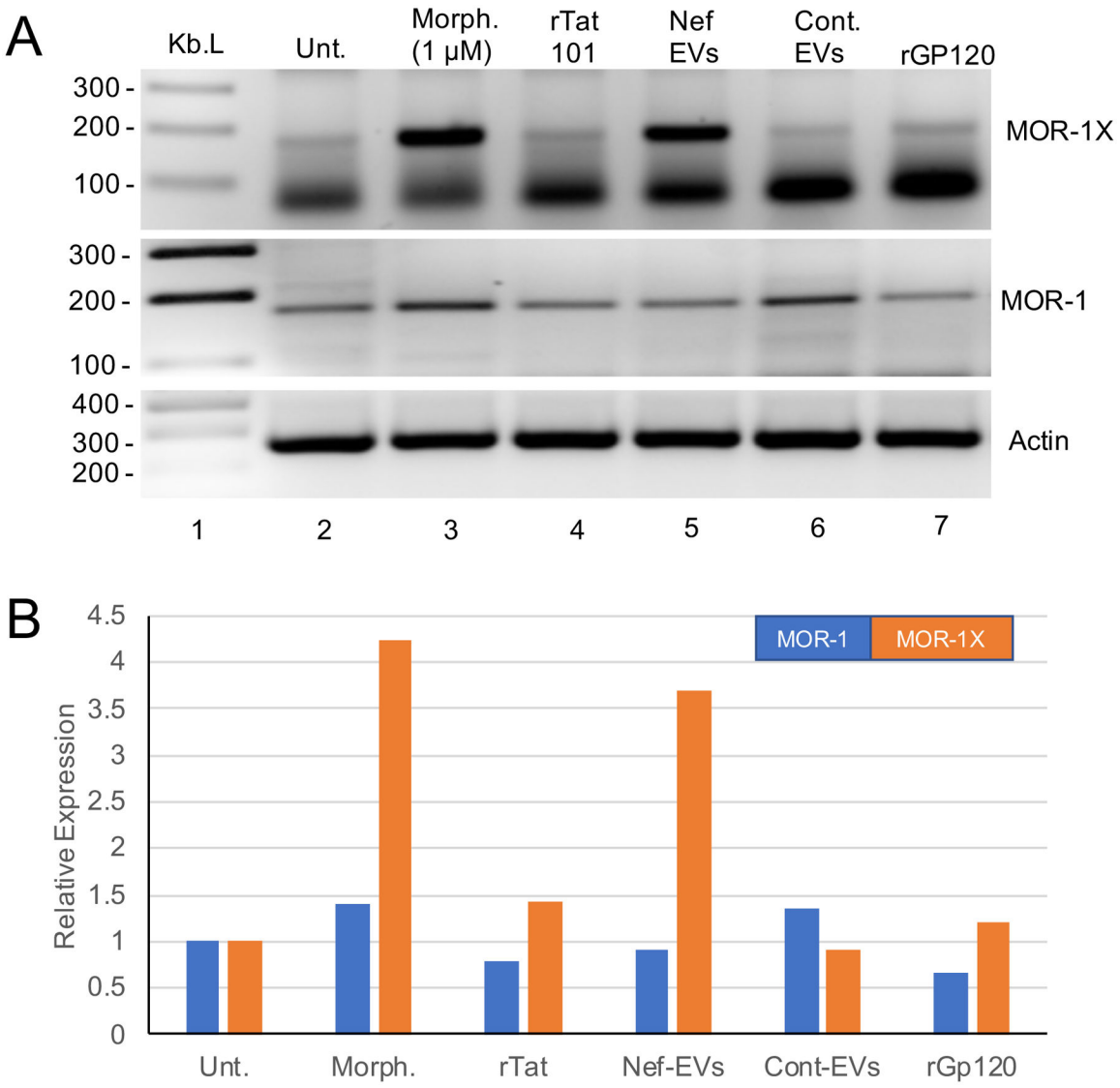

Figure 4: Nef-EVs induce alternative splicing of MOR-1X isoform in primary neurons:

A. Primary human neurons were treated with recombinant Tat-101 (100 ng/ml), recombinant gp120 (500 ng/ml), and Nef-EVs (1000 EV particles / cell). Cells were also treated with morphine (1 μM) and control EVs (1000 EVs / cell). Total RNA was extracted from the cells at 8 hrs post-treatments and analyzed by semi-quantitative RT-PCR for MOR-1 and MOR-1X isoforms. Beta-actin was also amplified in same samples and shown as internal control. B. MOR-1 and MOR-1X band intensities from the panel A were determined by Image J software and shown as relative expression in bar graph.

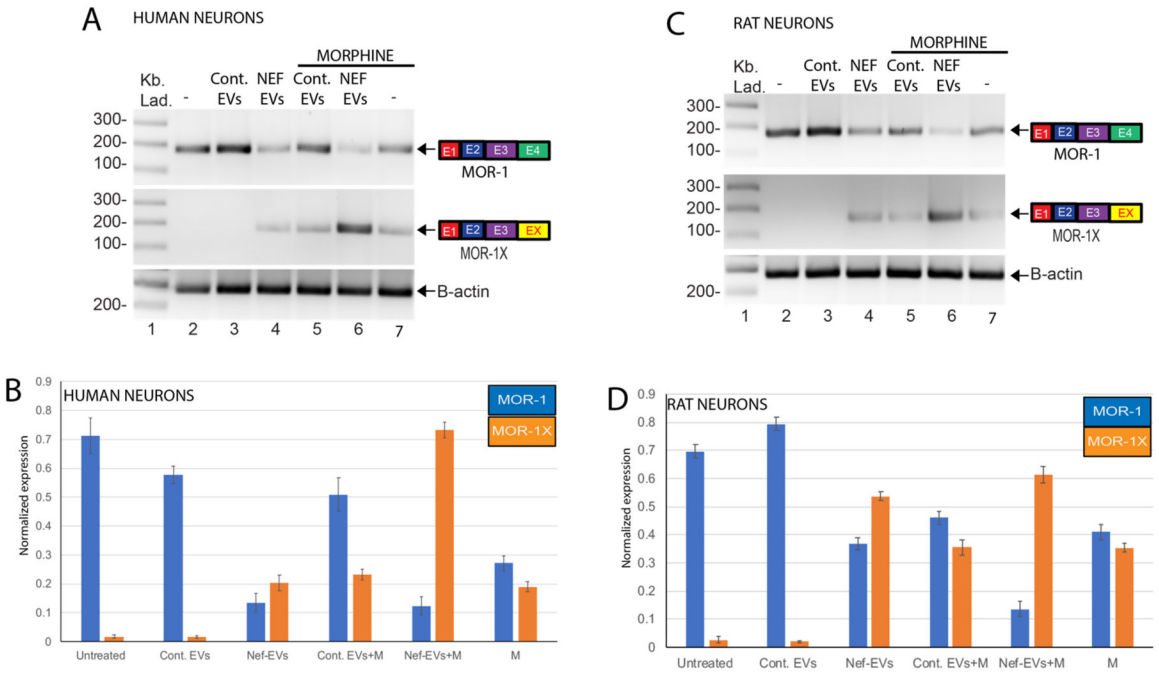

Figure 5. Effect of morphine and Nef-EVs co-treatment on MOR-1X alternative splicing in human and rat primary neurons.

Primary human (A) and rat (C) neurons were treated with either control EVs or Nef-EVs (500 EVs per cell) derived from primary astrocytes in the presence or absence of morphine (1uM). Total RNA was extracted from cells at 24hrs post treatments and processed by RT-PCR for the amplification of MOR-1 and MOR-1X isoforms. Beta-actin was also amplified from same samples. The exon/intron maps of each isoform and unique primer pairs used for differential amplification of both isoforms are schematized on top of the graphs. *B.* MOR-1 and MOR-1X band intensities from panel A were determined by Image J and shown as normalized expression in bar graph. D. MOR-1 and MOR-1X band intensities from panel C were determined by Image J and shown as normalized expression in bar graph. * SEM; $P<0.05$.

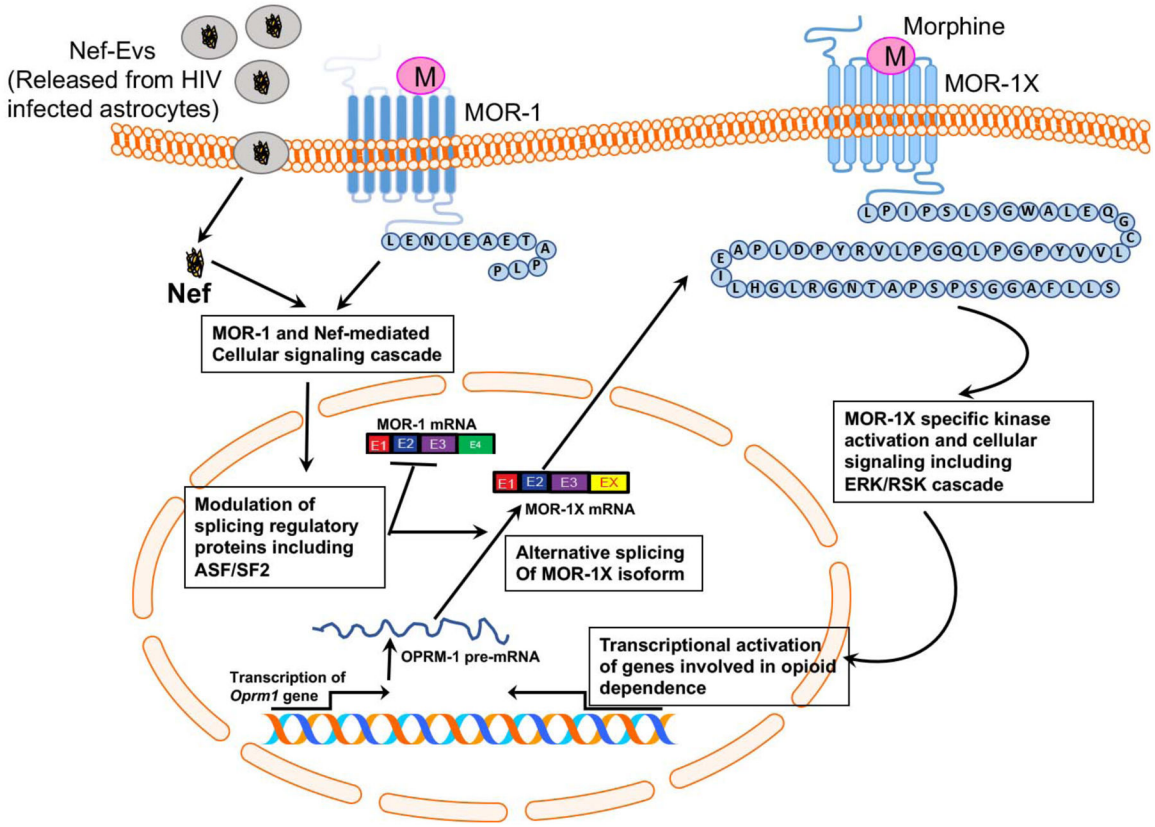

Figure 6.

Schematic illustration of a model summarizing the Nef-EVs and morphine effects on alternative splicing of OPRM1 pre-mRNA leading to induction of MOR-1X isoform and unique signaling for the expression of genes involved in opioid dependence.

Table 1:

Primer sequences used for semi-quantification of OPRM1 isoforms by RT-PCR.

PCR	NCBI Reference Sequence	Primer name	Sequence (5'→3')
Human OPRM1 Isoforms			
MOR-1X	NM_001008505.2*	F1	CCCAACCTCTTCCAACATTGAGCAA
		RMOR-1X	TCCCAGCCCGTCTGGTGGAG
		RMOR-1Xb	CTGTCTTGCTGGGCTCTAGAGC
MOR-1	NM_000914.5*	F2	ATGTCAGATGCTCAGCTCGGT
		RMOR-1/1i	CGGAGCAGTTTCTGCTTCC
		F1	CCCAACCTCTTCCAACATTGAGCAA
		RMOR-1	GCTCTAGAGCCCAGCAAGACAG
MOR-1i	NM_001145279.4*	F3	GCCCAGTGAAGAGACCTACTC
		RMOR-1/1i	CGGAGCAGTTTCTGCTTCC
MOR-1A	NM_001008504.4*	F1	CCCAACCTCTTCCAACATTGAGCAA
		RMOR-1A/1A2	AGAACCAGAGCAAGACTGG
MOR-1A2	NM_001285523.3*	F1	CCCAACCTCTTCCAACATTGAGCAA
		RMOR-1A/1A2	AGAACCAGAGCAAGACTGG
MOR-1B1	NM_001145282.2*	F1	CCCAACCTCTTCCAACATTGAGCAA
		RMOR-1B1	AGCCCTTGGTATGCTCACAGT
MOR-1B2	NM_001145283.2*	F1	CCCAACCTCTTCCAACATTGAGCAA
		RMOR-1B2/B3	GGCATGCTGGCAAAAGGGTA
MOR-1B3	NM_001145284.3*	F1	CCCAACCTCTTCCAACATTGAGCAA
		RMOR-1B2/B3	GGCATGCTGGCAAAAGGGTA
MOR-1B4	NM_001145285.3*	F1	CCCAACCTCTTCCAACATTGAGCAA
		RMOR-1B4/B5	AGGGTTCATGTCATAGTCAGCT
MOR-1B5	NM_001145286.3*	F1	CCCAACCTCTTCCAACATTGAGCAA
		RMOR-1B4/B5	AGGGTTCATGTCATAGTCAGCT
MOR-1O	NM_001008503.3*	F1	CCCAACCTCTTCCAACATTGAGCAA
		RMOR-1O	CGTGTAAAGATCTGGGCCATGC
Rat OPRM1 Isoforms			
MOR-1X	XM_008758731.2**	Fw	TCCACGATCGAACAGCAAAACT
		Rv	CAATGGAGCAGTTTCTGCCTCCAGAT
MOR-1	NM_013071.2**	Fw	TCGTCCACGATCGAACAGCAAAACT
		Rv	AAGGTTTAACATGATAGCTAAGTGTCTC
Reference genes			
GAPDH	NM_001289745.3* NM_017008.4**	Fw	ACCACAGTCCATGCCATCAC
		Rv	TCCACCACCCTGTTGCTGTA
β-Actin	NM_001101.5* NM_031144.3**	Fw	CTACAATGAGCTGCGTGTGGC
		Rv	CAGGTCCAGACGCAGGATGGC