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RESEARCH ARTICLE

Emotional Impairment and Persistent Upregulation of $mGlu_5$ Receptor following Morphine Abstinence: Implications of an $mGlu_5$-MOPr Interaction

Panos Zanos, PhD; Polymnia Georgiou, PhD; Loreto Rojo Gonzalez, BSc; Susanna Hourani, PhD; Ying Chen, PhD; Ian Kitchen, PhD; Brigitte L Kieffer, PhD; Raphaelle Winsky-Sommerer, PhD; Alexis Bailey, PhD

School of Biosciences and Medicine, Faculty of Health and Medical Sciences, University of Surrey, Guildford, Surrey, UK (Dr Zanos, Dr Georgiou, Ms Rojo Gonzalez, Prof. Hourani, Prof. Kitchen, Dr Winsky-Sommerer, and Dr Bailey); Department of Psychiatry, University of Maryland, School of Medicine, Baltimore, MD (Dr Zanos and Dr Georgiou); Institute of Psychiatry, Psychology and Neuroscience, Kings College London, London, UK (Dr Chen); Institut de Génétique et de Biologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, Université de Strasbourg, Illkirch, France (Prof. Kieffer); Douglas Hospital Research Center, Department of Psychiatry, Faculty of Medicine, McGill University, Montreal, Quebec, Canada (Prof. Kieffer); Institute of Medical and Biomedical Education, St George's University of London, London (Dr Bailey).

P.Z. and P.G. contributed equally to this work.

Correspondence: Alexis Bailey, PhD, Institute of Medical and Biomedical Education, St George's University of London, London SW17 0RE, UK (abailey@sgul.ac.uk).

Abstract

Background: A difficult problem in treating opioid addicts is the maintenance of a drug-free state because of the negative emotional symptoms associated with withdrawal, which may trigger relapse. Several lines of evidence suggest a role for the metabotropic glutamate receptor 5 in opioid addiction; however, its involvement during opioid withdrawal is not clear.
Methods: Mice were treated with a 7-day escalating-dose morphine administration paradigm. Following withdrawal, the development of affective behaviors was assessed using the 3-chambered box, open-field, elevated plus-maze and forced-swim tests. Metabotropic glutamate receptor 5 autoradiographic binding was performed in mouse brains undergoing chronic morphine treatment and 7 days withdrawal. Moreover, since there is evidence showing direct effects of opioid drugs on the metabotropic glutamate receptor 5 system, the presence of an metabotropic glutamate receptor 5/μ-opioid receptor interaction was assessed by performing metabotropic glutamate receptor 5 autoradiographic binding in brains of mice lacking the μ-opioid receptor gene.
Results: Withdrawal from chronic morphine administration induced anxiety-like, depressive-like, and impaired sociability behaviors concomitant with a marked upregulation of metabotropic glutamate receptor 5 binding. Administration of the metabotropic glutamate receptor 5 antagonist, 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine, reversed morphine abstinence-induced depressive-like behaviors. A brain region-specific increase in metabotropic glutamate receptor 5 binding was observed in the nucleus accumbens shell, thalamus, hypothalamus, and amygdala of μ-opioid receptor knockout mice compared with controls.

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Conclusions: These results suggest an association between metabotropic glutamate receptor 5 alterations and the emergence of opioid withdrawal-related affective behaviors. This study supports metabotropic glutamate receptor 5 system as a target for the development of pharmacotherapies for the treatment of opioid addiction. Moreover, our data show direct effects of μ-opioid receptor system manipulation on metabotropic glutamate receptor 5 binding in the brain.

Keywords: Morphine, withdrawal, μ-opioid receptor, $mGlu_5R$, opioids

Introduction

Opioid addiction is a relapsing brain disorder characterized by compulsion to seek and take the drug, despite the negative physical and emotional consequences following abstinence (Le Moal and Koob, 2007). Symptoms associated with affective emotional impairment, including anxiety, depression, stress, and anhedonia, during drug withdrawal act as a motivational trigger to relapse (Martin and Jasinski, 1969; Jaffe, 1990; Nunes et al., 2004; Peles et al., 2007), which is the primary problem for the treatment of opioid addiction (Stotts et al., 2009). In fact, there is 30% to 40% comorbidity between substance use disorders and illicit drug abuse with major depression (Davis et al., 2008). Addressing the mechanisms underlying the emotional impairment associated with opioid abstinence is essential given this high comorbidity prevalence, along with the ambiguous efficacy of classic antidepressant treatment in this patient population (Nunes et al., 2004).

While opioid drugs, including morphine and heroin, primarily act on the μ-opioid receptor (MOPr) to exert their neurochemical and behavioral effects (Matthes et al., 1996), evidence also suggests a role for the glutamatergic system to underlie several opioid addiction processes (Kalivas, 2009). A role for metabotropic glutamate receptor 5 ($mGlu_5R$) in drug self-administration as well as reinstatement of drug-seeking behavior has been reported (Gass and Olive, 2008). With respect to opioids, pharmacological blockade of the $mGlu_5R$ attenuated the development of tolerance to morphine (Kozela et al., 2003; Gabra et al., 2008), inhibited the expression of morphine locomotor sensitisation (Kotlinska and Bochenski, 2007), prevented the acquisition (Roohi et al., 2014) and expression of morphine place-preference (Popik and Wrobel, 2002; Aoki et al., 2004; Herzig and Schmidt, 2004; Veeneman et al., 2011), and decreased morphine self-administration (Brown et al., 2012) in rodents. In addition, administration of the $mGlu_5R$ antagonist, 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine (MTEP), decreased cue-induced reinstatement of morphine seeking in mice (Brown et al., 2012), and intra-nucleus accumbens shell (AcbSh) administration of the $mGlu_5R$ antagonist 2-Methyl-6-(phenylethynyl)pyridine (MPEP) decreased heroin-seeking induced by context, cues, or heroin priming in rats (Lou et al., 2014). Moreover, $mGlu_5R$ antagonism has been found to potentiate the antinociceptive effects of morphine in rats (Picker et al., 2011). However, the role of the $mGlu_5R$ on the modulation of morphine withdrawal-induced emotional impairment has not yet been investigated.

Therefore, in the present study, we first aimed to characterize the behavioral/emotional consequences of morphine withdrawal and investigate the effects of chronic morphine administration and withdrawal on $mGlu_5R$ binding in the brain of mice. We found that morphine abstinence induced anxiety-like, depressive-like, and impaired sociability behaviors concomitant with a marked region-specific upregulation of $mGlu_5R$ binding, possibly suggesting the presence of MOPr-$mGlu_5R$ interaction in the brain. We also assessed the effects of $mGlu_5R$ antagonism on the reversal of morphine withdrawal-induced depressive-like behaviors to directly explore the possible involvement of central $mGlu_5R$ system in the emotional component of morphine withdrawal. To further assess the effects of MOPr system manipulation on the $mGlu_5R$ system, we performed $mGlu_5R$ autoradiographic binding in brains of mice lacking the MOPr gene (MOPr knockout mice). Our findings provide further evidence for a key role of the central $mGlu_5R$ system in the modulation of the emotional consequences of opioid abstinence and suggest the existence of an $mGlu_5R$-MOPr interaction to be localized in the nucleus accumbens, thalamus, hypothalamus, and amygdala, regions highly associated with reward and emotions.

Methods

Animals

Chronic Morphine and Withdrawal Experiments—Male C57BL/6J mice (7 weeks old, 20–25 g, Charles River Laboratories, Kingston, UK) were housed individually in a temperature-controlled environment with a 12-hour-light/-dark cycle (lights on 6:00 AM). Food and water were available ad libitum. Mice acclimatized in their new environment for at least 7 days prior to experiments and were handled daily. All experimental procedures were conducted in accordance with the UK Animal Scientific Procedures Act (1986).

Mu Opioid Receptor Knockout Study—The brains from 8-week-old MOPr knockout mice were provided by Professor Brigitte Kieffer's laboratory (IGBMC, Strasbourg, France). The experimental methodology for the generation of MOPr knockout mice used in the current study has been previously described elsewhere (Matthes et al., 1996).

Chronic "Intermittent" Escalating-Dose Morphine Administration Paradigm and Withdrawal

Mice were randomly divided into chronic saline- and morphine-treated groups (n = 6/group). Mice were injected (i.p.) with saline (4 mL/kg) or morphine-HCl (Sigma-Aldrich, Poole, UK) with a chronic, "intermittent" escalating-dose administration paradigm (20 mg/kg on day 1, 40 mg/kg on days 2 and 3, 80 mg/kg on days 4 and 5, and 100 mg/kg on days 6 and 7) twice per day at 9:00 AM and 5:00 PM in accordance with previously published protocols (Muller and Unterwald, 2004; Zhou et al., 2006; Goeldner et al., 2011; Zanos et al., 2014). Both groups of animals were left to spontaneously withdraw in their home cages without injections. Different cohorts of mice were assessed for withdrawal-associated affective behaviors and $mGlu_5R$ binding. Body weight was measured daily.

Characterization of a Mouse Model of Opioid Withdrawal

To characterize the behavioral effects of morphine withdrawal, mice were treated with 7 days of escalating-dose morphine

paradigm and spontaneous withdrawal for a period of 6 to 8 days. Mice were tested for sociability (6 days withdrawal; Crawley's 3-chambered social approach test), anxiety-like behavior (7 days withdrawal; open-field and elevated-plus maze [EPM] tests), and depression-like behaviors (8 days withdrawal; forced-swim test [FST]). The order of testing was determined by the degree of stress-inducing properties of each test, with the least stressful conducted first and the most potentially distressing test last (Clemens et al., 2007). The Crawley's 3-chambered social approach test, EPM, and FST were performed as previously described (Zanos et al., 2014) (see supplementary Materials and Methods). To further assess opioid abstinence-associated anxiety-related behaviors, mice were placed in open-field arenas (40 cm x 20 cm x 20 cm) 7 days following the last treatment injection, and time spent in the center of the arena was scored for 10 minutes by a trained observer blind to the treatment groups. Twenty-four hours following the last behavioral test (ie, FST), mice were euthanized by 30-second exposure to CO_2 followed by decapitation and spleen was weighed for peripheral assessment of stress (Kyo et al., 1999; Dominguez-Gerpe and Rey-Mendez, 2001).

To assess the role of $mGlu_5R$ on morphine withdrawal-induced depressive-like behaviors, we used a different cohort of mice undergoing the same morphine administration and withdrawal paradigm as described above. On day 8 (24 hours following a 15-minute FST pretest) of withdrawal, we administered saline (vehicle) or MTEP (3 mg/kg, i.p.), and 30 minutes later we assessed for depressive-like behaviors in the FST, as described by Zanos et al. (2014). Dose and timing of administration of MTEP was determined based on previous studies assessing the effects of MTEP in the FST paradigm (Palucha et al., 2005; Belozertseva et al., 2007; Pomierny-Chamiolo et al., 2010; Domin et al., 2014).

Autoradiographic Binding of $mGlu_5R$ in the Brain of Mice

A separate cohort of mice underwent the same administration paradigm for biochemical assessments. For this cohort of animals, mice were not assessed in any other behavioral test. One hour following the final morphine/saline injection for the chronic administration group and 7 days postfinal injection for the withdrawal groups, mice were euthanized by 30-second exposure to CO_2 followed by decapitation and brains were collected and immediately frozen in isopentane solution (-25°C). Coronal brain sections were cut (20 μm thick; 300 μm apart) using a cryostat (Zeiss Microm 505E, Hertfordshire, UK), thaw-mounted onto gelatin subbed ice-cold microscope slides, and processed for autoradiography as described previously (Kitchen et al., 1997).

Quantitative $mGlu_5R$ autoradiography was performed in brain sections of all treatment groups and both genotypes as described previously (Georgiou et al., 2015; Wright et al., 2015). For the determination of total binding, slides were incubated for 60 minutes in 10 nM [^{3}H]-MPEP (American Radiolabeled Chemical, 2.22 TBq/mmol) in Tris-HCl buffer (pH 7.4, 4°C). Adjacent brain section were incubated with [^{3}H]-MPEP (10 nM) in the presence of 10 μM fenobam (Tocris Bioscience, Bristol, UK) to determine the nonspecific binding.

Slides with radioligand bound sections were apposed to films (Kodak BioMax MR-1 films; Sigma-Aldrich, Gillingham, UK) for 3 weeks along with appropriate ^{3}H microscale standards (Amersham Pharmacia Biotech) to allow quantification. Films were then developed and analyzed in parallel in a complete paired protocol as described by Kitchen et al. (1997). All structures were identified by reference to the mouse brain atlas of Franklin & Paxinos (2007) and analyzed using an image analyzer (MCID; Image Research, Linton, UK).

Statistical Analysis

All the values are expressed as mean ± SEM. The effects of morphine withdrawal on anxiety-like and depressive-like behaviors were analyzed by Student's *t* test. For analysis of sociability deficits, 2-way repeated-measures ANOVA was performed with factors "treatment" and "chamber (ie, empty and stranger – repeated factor)." The effects of morphine administration and withdrawal on spleen weight were analyzed by 2-way ANOVA for factors "treatment" (ie, saline and morphine) and "experimental phase" (ie, chronic and withdrawal). For analysis of $mGlu_5R$ autoradiographic binding following chronic morphine administration and withdrawal, 2-way ANOVA with factors "treatment" (ie, saline and morphine) and "experimental phase" (ie, chronic and withdrawal) was performed in each brain region. The effects of MTEP pretreatment on depressive-like behaviors in the FST were assessed using 2-way ANOVA for factors "pretreatment" (ie, saline and MTEP) and "treatment" (ie, saline and morphine). For assessing the effects of MOPr gene deletion on $mGlu_5R$ binding, unpaired Student's *t* test was used in each individual region. For the regression analysis, Pearson correlation coefficient test was performed. ANOVAs were followed by a Bonferoni post-hoc test when significant interaction effect was reached (ie, $P < .05$). All statistical analyses were performed using SigmaPlot v11.0 (Systat Software, London, UK).

Results

Effect of Morphine Withdrawal on Anxiety-Like, Depressive-Like, and Sociability Behaviors

Anxiety-Like Behaviors—We assessed anxiety-like behavior following the 7-day withdrawal from morphine using the EPM and open-field tests. Morphine-withdrawn animals spent significantly less time in the open arms ($P < .01$; Figure 1*a*) and showed decreased time in the centre of the open-field arena ($P < .01$; Figure 1*b*) compared with saline-withdrawn animals.

Depressive-Like Behavior—Depressive-like behavior following the 8-day withdrawal from morphine was assessed using the FST. Morphine-withdrawn animals showed significantly increased immobility time compared with saline-withdrawn animals ($P < .05$) (Figure 1*c*). Morphine-withdrawn mice needed significantly less time to reach the first immobility compared to saline-withdrawn mice ($P < .01$) (Figure 1*d*).

Sociability—The social preference in morphine-withdrawn mice was assessed using the 3-chambered sociability test following 6 days of withdrawal. Saline-withdrawn mice exhibited a significant preference for the chamber containing stranger mouse vs the empty chamber ($P < .001$) (Figure 1*e*). However, morphine-withdrawn animals did not show any preference between the chamber containing the stranger mouse and the empty chamber ($P > .05$; Figure 1*e*), demonstrating a lack of social preference. To directly demonstrate a lack of social preference of morphine withdrawn mice, we further analyzed the ratio of time spent in the "stranger" side vs time spent in the chamber containing the empty cage (social interaction ratio = time in stranger side/time in empty cage chamber). This analysis revealed a strong preference for saline control mice to spend their time in the social chamber (2.14 ± 0.23 vs threshold of 1; 1-sample *t* test; $P < .001$), whereas morphine withdrawn mice did not show preference

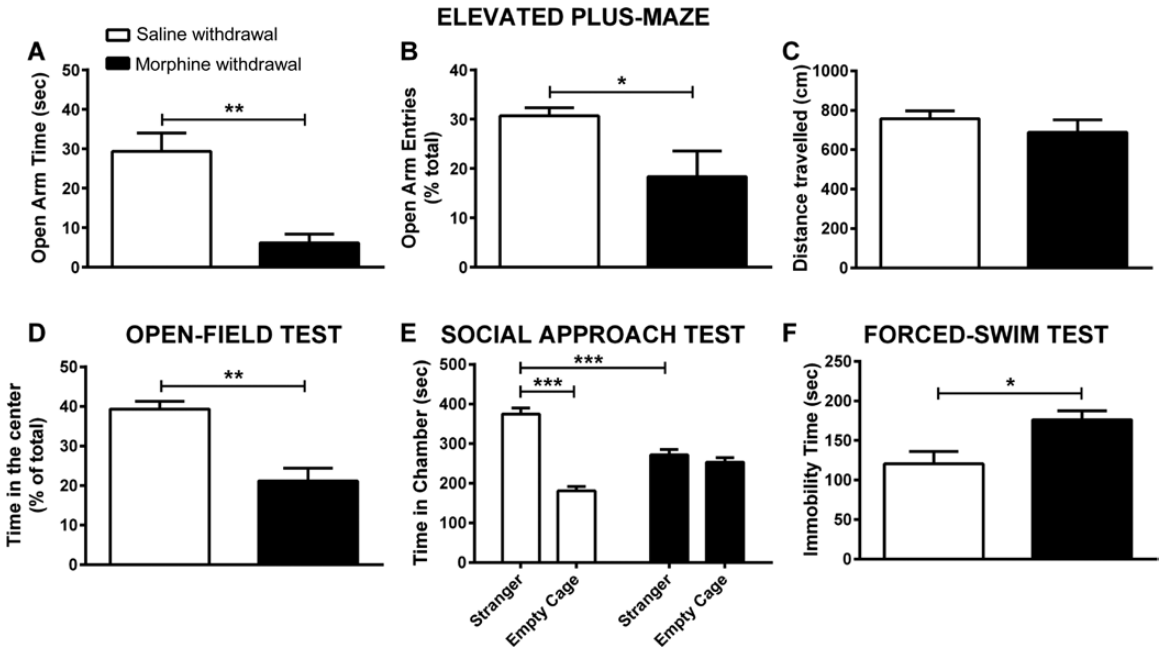

Figure 1. Depressive-like, anxiety-like behaviors, and sociability deficits following chronic protracted morphine withdrawal. Male C57BL/6J mice were treated with either saline or an escalating-dose morphine paradigm (2 x 20–100 mg/kg/d, i.p.) followed by 6 to 8 days withdrawal. (A) Open-arm time. (B) Open-arm entries. (C) Distance travelled in the elevated plus-maze (EPM) and (D) time in the center (percent of total) of an open field (OF) were measured to assess anxiety-like behaviors following 6 and 7 days of morphine withdrawal, respectively. (E) Sociability of morphine- and saline-withdrawn mice was also assessed using Crawley's 3-chambered social approach test. (F) Immobility time was assessed in the forced-swim test (FST) following 7 days morphine withdrawal. Data are expressed as the mean ± SEM (n = 6/group). *P < .05, **P < .01, ***P < .001 (EPM, OF, and FST behaviors were scored using the Student's *t* test; sociability behavior was analyzed using repeated-measures 2-way ANOVA followed by Bonferroni post-hoc test).

for either of the 2 compartments (1.09 ± 0.09 vs threshold of 1; 1-sample *t* test; $P > .05$).

Effect of Chronic Morphine Administration and Withdrawal on Body Mass and Relative Spleen Weight

Body weight was measured daily throughout the chronic morphine administration and withdrawal paradigm. Chronic administration of morphine resulted in a lack of body weight gain compared with saline-treated animals, which reached statistical significance on day 5 ($P < .01$) (Figure 2*a*). Decreased weight gain persisted up to the second day of withdrawal (ie, day 9) (day 6, $P < .01$; day 7, $P < .001$; day 8, $P < .05$; day 9, $P < .01$ vs saline controls) (Figure 2*a*).

We measured spleen weight in animals undergoing chronic morphine administration as well as withdrawal from morphine as an indicator of stress state of the animal. Indeed, decreased spleen weight has been previously associated with stress in rodents (Kyo et al., 1999; Dominguez-Gerpe and Rey-Mendez, 2001). Seven days of morphine administration did not change the relative spleen weight compared with the saline-treated animals. However, the 7-day withdrawal period from morphine administration induced a significant decrease in relative spleen weight compared with the saline-withdrawn controls ($P < .001$) (Figure 2*b*).

Effect of Chronic Morphine Administration and Withdrawal on $mGlu_5R$ Binding

Quantitative analysis of $mGlu_5R$ binding of morphine treated and withdrawal animals revealed a significant treatment effect in all the brain regions analyzed, including prelimbic cortex ($F_{(1,20)} = 21.370$; $P < .001$), AcbC ($F_{(1,22)} = 94.810$; $P < .001$), AcbSh ($F_{(1,22)} = 74.450$; $P < .001$), lateral septum ($F_{(1,20)} = 37.160$; $P < .001$), medial septum ($F_{(1,22)} = 37.310$; $P < .001$), caudate putamen ($F_{(1,20)} = 61.160$; $P < .001$), hippocampus ($F_{(1,23)} = 19.850$; $P < .001$), amygdala ($F_{(1,23)} = 13.540$; $P < .01$), thalamus ($F_{(1,23)} = 13.170$; $P < .01$), hypothalamus ($F_{(1,23)} = 13.60$; $P < .01$), visual cortex ($F_{(1,24)} = 6.498$; $P < .05$), ventral tegmental area ($F_{(1,24)} = 10.490$; $P < .01$), superficial grey ($F_{(1,24)} = 8.356$; $P < .01$), and periaqueductal grey ($F_{(1,24)} = 6.188$; $P < .05$). A significant treatment x experimental phase interaction effect was observed in prelimbic cortex ($F_{(1,20)} = 9.612$; $P < .001$), AcbC ($F_{(1,22)} = 21.760$; $P < .001$), AcbSh ($F_{(1,22)} = 14.180$; $P < .01$), lateral septum ($F_{(1,20)} = 20.114$; $P < .001$), medial septum ($F_{(1,22)} = 10.120$; $p < 0.01$), caudate putamen ($F_{(1,20)} = 16.280$; $P < .001$), thalamus ($F_{(1,23)} = 5.625$; $P < .05$) and hypothalamus ($F_{(1,23)} = 4.330$; $P < .05$). Following the 7-day morphine administration, Bonferroni post-hoc analysis showed a significant increase in $mGlu_5R$ binding in the AcbC ($P < .001$), AcbSh ($P < .01$), and caudate putamen ($P < .01$) compared with the respective saline control (Figure 3*a*-*b*). Notably, the increase in $mGlu_5R$ binding observed in these regions was further enhanced following the 7-day withdrawal period (chronic morphine compared with morphine withdrawal; $P < .001$) (Figure 3*a*-*b*). A morphine-withdrawal effect was observed in all the brain regions analyzed (Figure 3*a*-*b*; prelimbic cortex, $P < .001$; lateral septum, $P < .001$; medial septum, $P < .001$; thalamus, $P < .001$; hypothalamus, $P < .001$) compared with the respective saline-withdrawn controls. Compared with chronic morphine treatment, morphine withdrawal induced an upregulation of the $mGlu_5R$ binding in the prelimbic cortex ($P < .01$), medial septum ($P < .01$), lateral septum ($P < .001$), thalamus ($P < .001$), and hypothalamus ($P < .01$).

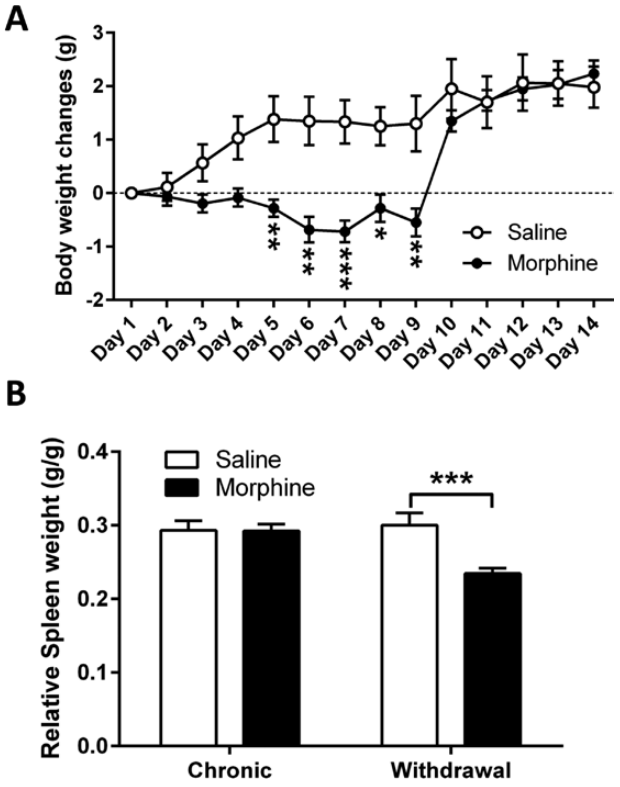

Figure 2. Effects of chronic morphine administration and withdrawal on body weight and relative spleen weight. Male C57BL/6J mice were treated with either saline or escalating-dose morphine paradigm (2 x 20–100 mg/kg/d, i.p.) followed by a 7-day withdrawal period. (A) Changes in body weight and (B) relative spleen weight during chronic morphine or saline administration and withdrawal. Data are expressed as the mean ± SEM (n=6–12/group). *P<.05, **P<.01, ***P<.001 vs saline control (repeated-measures 2-way ANOVA followed by Bonferroni post-hoc test).

Effect of Acute Administration of the $mGlu_5R$ Antagonist MTEP on Morphine Withdrawal-Induced Depressive-Like Behaviors

Administration of MTEP 30 minutes prior to the FST reversed the increased immobility time induced by 8 days of morphine withdrawal in mice (Figure 4). Two-way ANOVA revealed a significant main effect of treatment ($F_{(1,19)}$= 10.230; $P<.01$) and pre-treatment x treatment interaction ($F_{(1,23)}$=4.66; $P<.05$). Morphine-withdrawn animals manifested increased immobility time in the FST, indicating the emergence of a depressive-like phenotype, while MTEP pretreatment abolished this behavioral deficit induced by morphine abstinence. MTEP administration did not alter immobility time of control/saline-treated animals (Figure 4).

Effects of MOPr Gene Deletion on $mGlu_5R$ Binding

Quantitative autoradiography of $mGlu_5$ receptors from coronal sections of fore-, mid-, and hind-brain mice showed a widespread distribution. The pattern of distribution of $mGlu_5$ receptors on MOPr knockout mice brains was identical to the wild-type mouse brains (Figure 5*a*). MOPr knockout brains had significantly increased $mGlu_5R$ binding in the AcbSh ($P<.05$), thalamus ($P<.01$), hypothalamus ($P<.01$), and amygdala ($P<.05$) (Figure 5*b*).

Regression analysis was carried out to determine if there was a correlation between the MOPr binding levels in regions of wild-type brains and percent change in $mGlu_5R$ binding in the regions analyzed of MOPr knockout mouse brains compared with MOPr binding levels derived from wild-type mice (Kitchen et al., 1997). Regression analysis revealed a significant correlation (r=0.7, n=14, $P<.01$) (Figure 5*c*).

Discussion

The present study is the first, to our knowledge, to demonstrate a significant upregulation of $mGlu_5R$ throughout the whole brain in a mouse model of opioid-withdrawal characterized by emotional impairment. We showed that a 6- to 8-day withdrawal period from chronic morphine administration induced anxiety-like and depressive-like behaviors, and impaired sociability. Moreover, we provide evidence for direct effects of a MOPr system manipulation on the $mGlu_5R$ in the AcbSh, thalamus, hypothalamus, and amygdala, which are brain regions important in drug addiction and emotional regulation (Bossert et al., 2007; Chaudhri et al., 2010; Zanos et al., 2014).

We first characterized a mouse model of opioid withdrawal, which mimics the negative affective state displayed by opioid addicts undergoing abstinence following detoxification (Martin and Jasinski, 1969; Jaffe, 1990; Nunes et al., 2004; Peles et al., 2007; Veilleux et al., 2010). We have previously shown, using the same chronic morphine administration and withdrawal protocol, that physical withdrawal symptoms associated with acute morphine withdrawal (ie, withdrawal jumping, increased defecation, and changes in body weight) disappear following a 7-day abstinence period (Goeldner et al., 2011; Zanos et al., 2014); however, emotional impairment is evident, highlighting the translational validity of our mouse model. This impairment has been reported to be even more evident after 4 weeks of abstinence (Goeldner et al., 2011) and was accompanied by significant alterations of gene expression in the extended amygdala (Le Merrer et al., 2012). In the present study, decrease in relative spleen weight was observed in 7-day-withdrawn animals, further supporting a stress-related phenotype. Decreased spleen weight has been previously correlated with higher levels of emotional stress in rodents (Kyo et al., 1999; Dominguez-Gerpe and Rey-Mendez, 2001).

The emotional impairment observed in the present study was accompanied by an abundant increase in $mGlu_5R$ binding in the brain. These findings are supported by previous data showing that chronic morphine administration induces a dose-dependent upregulation of $mGlu_5R$ immunoreactivity in the spinal cord in mice (Narita et al., 2005). An increase in the expression of $mGlu_5R$ protein was also found in the spinal cord of morphine-tolerant rats (Liu et al., 2009) and the ventral pallidum of morphine-dependent rats (Herrold et al., 2013). Interestingly, administration of the $mGlu_5R$ antagonist MPEP prevented the development of morphine tolerance by specifically preventing the increased expression of $mGlu_5R$ protein in the spinal cord (Liu et al., 2009), supporting a biological/behavioral relevance of these $mGlu_5R$ alterations. However, this is first study to demonstrate profound alterations of $mGlu_5R$ binding in the brain following opioid withdrawal.

Although the molecular mechanism underlining the chronic morphine/withdrawal induced upregulation of $mGlu_5R$ is not clear, one can speculate on various potential mechanisms. First, there is evidence to suggest that $mGlu_5R$ upregulation may be a result of a compensatory neuroadaptive response to a suppression of glutamate neurotransmission during morphine withdrawal. Indeed there is evidence showing that chronic opioid administration suppresses glutamate neurotransmission

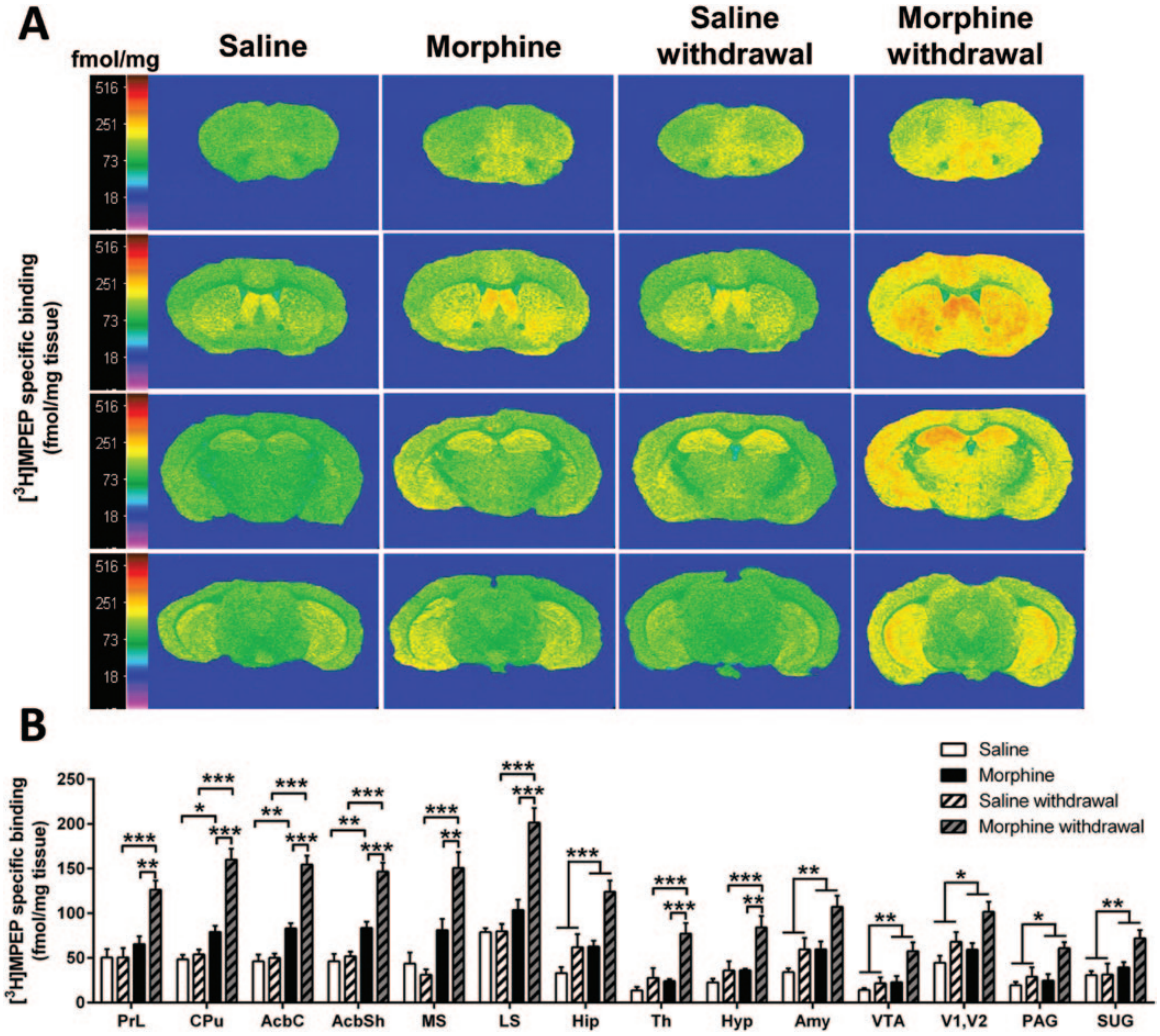

Figure 3. Morphine withdrawal increases metabotropic glutamate receptor 5 ($mGlu_5R$) binding in the brain. (A) Representative autoradiograms of 10 μM [^{3}H]-2-methyl-6-([3,5-3H] phenylethynl) pyridine ([^{3}H]MPEP) binding to $mGlu_5R$ in coronal brain sections of mice undergoing chronic saline or morphine administration and 7-day withdrawal. Autoradiograms of brain sections were taken at the level of prefrontal cortex (Bregma: 2.34 mm; first row), striatum (Bregma: 0.62 mm; second row), thalamus (Bregma: -1.82 mm; third row), and ventral tegmental area (Bregma: -3.40 mm; fourth row). Binding levels are represented using a pseudocolor interpretation of black and white film images in fmol/mg of tissue equivalent. (B) Quantitative $mGlu_5R$ binding in the brain of mice subjected to morphine administration and withdrawal. Data are expressed as the mean ± SEM (n = 6–7/group). *$P < .05$, **$P < .01$, ***$P < .001$ (2-way ANOVA followed by Bonferroni post-hoc test per brain region).

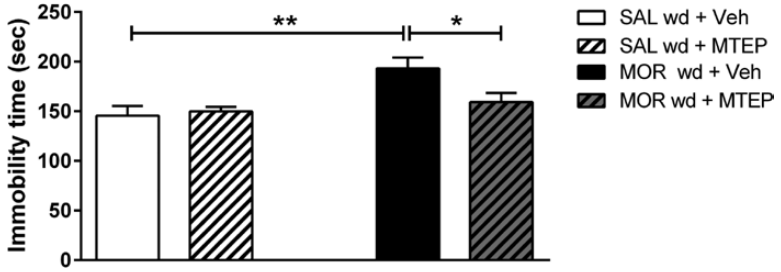

Figure 4. Pretreatment with the metabotropic glutamate receptor 5 ($mGlu_5R$), 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine (MTEP), prevents morphine abstinence-induced depressive-like behaviors. Male C57BL/6J mice were treated with either saline or escalating-dose morphine paradigm (2 x 20–100 mg/kg/d, i.p.) followed by a 7-day withdrawal period. Administration of the $mGlu_5R$ antagonist, MTEP, abolished the depressive-like behaviors induced by morphine withdrawal in the forced-swim test (FST). Data are expressed as the mean ± SEM (n = 5–6/group). *$P < .05$, **$P < .01$ (2-way ANOVA followed by Bonferroni post-hoc test).

in the central nervous system (Manzoni and Williams, 1999; Xu et al., 2003). This hypoglutamatergic state may thus in turn induce a compensatory increase in $mGlu_5R$ to enhance glutamatergic synaptic transmission. It has been postulated that such a mechanism may at least partly be responsible for the suppression of tolerance to spinal antinociception induced by morphine treatment (Suzuki et al., 2006). Another potential mechanism may involve chronic morphine-induced allosteric modulation of $mGlu_5R$. Indeed, Schroder et al. (2009) provided compelling evidence that this may be the case in vitro. They

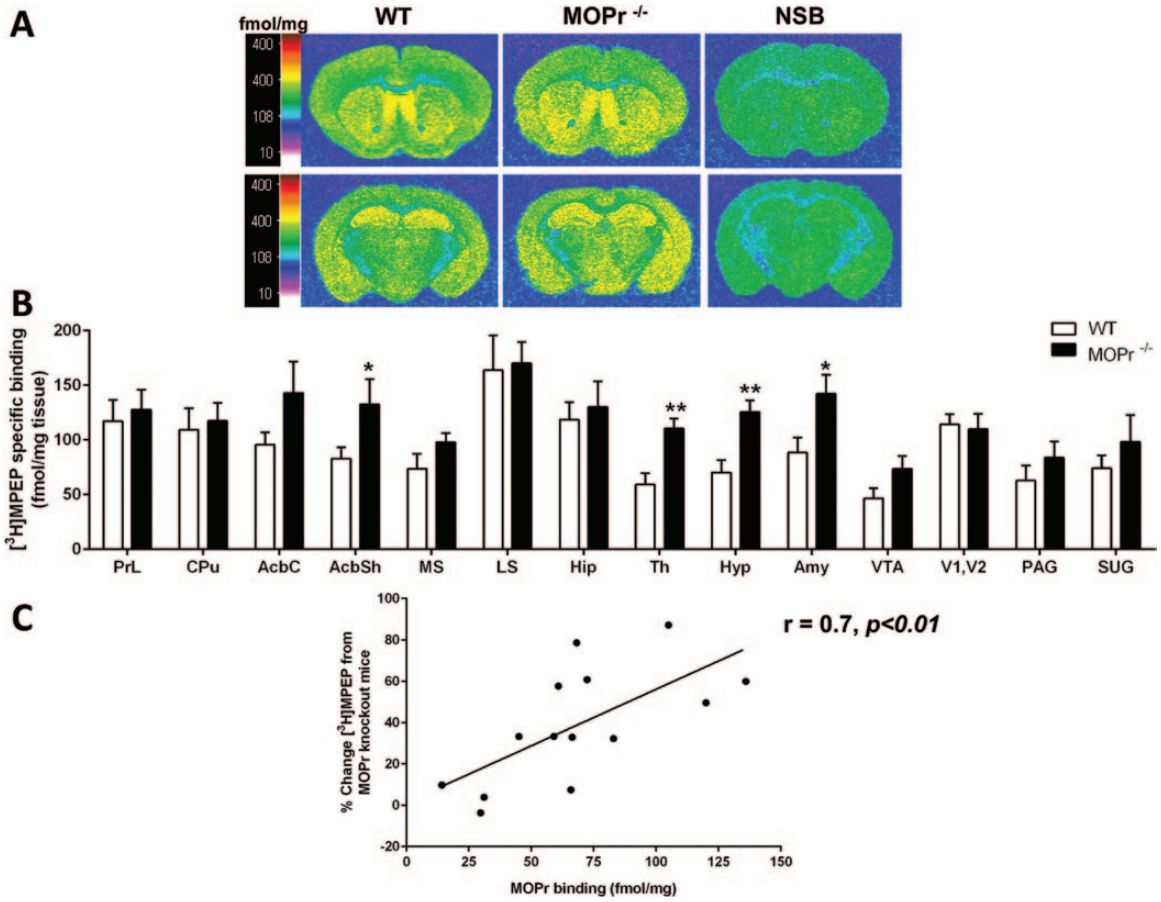

Figure 5. Metabotropic glutamate receptor 5 ($mGlu_5R$) binding in the brain of mice lacking the μ-opioid receptor (MOPr) gene. (A) Representative autoradiograms of 10 μM [^{3}H]-2-methyl-6-([3,5-3H] phenylethynl) pyridine ([^{3}H]MPEP) binding to $mGlu_5R$ in coronal brain sections of wild-type (WT) and MOPr knockout ($MOPr^{-/-}$) mice. Autoradiograms of brain sections were taken at the level of striatum (Bregma: 0.62 mm; first row) and thalamus (Bregma: -1.82 mm; second row). Representative autoradiograms for nonspecific binding (NSB) are shown (far right column). Binding levels are represented using a pseudocolor interpretation of black and white film images in fmol/mg of tissue equivalent. (B) Quantitative $mGlu_5R$ binding. Data are expressed as the mean ± SEM (n = 7/group). *P < .05, **P < .01, ***P < .001 vs wild-type control (Student's *t*-test per brain region). (C) Correlation analysis between percentage change in $mGlu_5R$ binding in MOPr knockout mice and MOPr binding in wild-type mice. (n = 14; r = 0.7; P < .01).

found that MPEP binding site affinity and Bmax for $mGlu_5R$ is significantly increased in MOPr- $mGlu_5R$ coexpressing cells vs $mGlu_5R$-only expressing cells, suggesting that the interaction between MOPr and $mGlu_5R$ can profoundly alter the conformation of $mGlu_5R$. In our study, it cannot be determined if chronic morphine/withdrawal alters the affinity or Bmax of $mGlu_5R$ or both, but based on the evidence of Schroder et al. (2009), it is tempting to speculate that the profound upregulation of $mGlu_5R$ in response to chronic morphine treatment could be caused by a change in $mGlu_5R$ affinity and/or decrease in receptor internalization. Finally, there is increasing evidence suggesting the existence of MOPr-$mGlu_5R$ heterodimerization that may at least partly explain the profound changes of $mGlu_5R$ following chronic morphine treatment/withdrawal. Indeed, studies showing that desensitization, phosphorylation and internalization of MOPr are influenced by mGlu5R modulation (Schroder et al., 2009), further suggest the presence of MOPr-mGlu5R heterodimers. Furthermore, it has been demonstrated that targeting the putative MOPr-$mGlu_5R$ heteromer using a bivalent ligand containing MOPr agonist and $mGlu_5R$ antagonist is effective in decreasing inflammatory and chronic cancer pain (Akgun et al., 2013; Smeester et al., 2014).

To directly assess the relation between the observed upregulation of the $mGlu_5R$ and the behavioral deficits of morphine-withdrawn mice, we tested the effect of an $mGlu_5R$ receptor antagonist, MTEP, on morphine abstinence-induced depressive-like behavior in the FST. The complete reversal of morphine abstinence-associated depressive-like behavior following administration of MTEP clearly indicates a key role for $mGlu_5R$ in the manifestation of the emotional consequences of opioid abstinence and is highly suggestive of a causal relationship between the evident $mGlu_5R$ upregulation in the brain of morphine-withdrawn mice and the observed emotional deficits. This finding is consistent with the antidepressant effect of $mGlu_5R$ antagonists in rodent stress models (Li et al., 2006; Belozertseva et al., 2007). It is also likely that $mGlu_5R$ may also be involved in the anxiogenic and social deficit consequences of opioid abstinence, as $mGlu_5R$ antagonism was shown to reverse ethanol withdrawal-induced anxiety (Kumar et al., 2013) and rescue social deficits observed in a mouse model of autism (Silverman et al., 2012).

To further explore possible effects of a manipulated MOPr system on the $mGlu_5R$ in the brain, we assessed the effect of MOPr gene deletion on $mGlu_5R$ binding with the use of MOPr knockout mice, which are characterized by certain behavioral phenotypic and MOPr signalling characteristics that are reminiscent of our model of morphine abstinence. Notably, similar to our morphine-withdrawal mouse model, MOPr knockout mice display deficits

in social behavior and emotional impairment (Becker et al., 2014). Furthermore, consistent with the MOPr KO mouse model, where MOPr are completely absent, repeated administration of morphine, which primarily acts on MOPr, is well known to induce a significant reduction in MOPr activation of G-protein in the brain of rats (Sim et al., 1996) and thus lead to MOPr desensitization that persists during withdrawal (Ingram et al., 2007). Therefore, it is not perhaps surprising that similar alterations of $mGlu_5R$ binding in the same direction are observed in brain regions of MOPr KO mice and chronically morphine-treated/-withdrawn mice, both of which are models of reduced MOPr activity.

Our data demonstrate a marked $mGlu_5R$ upregulation in MOPr-null mice in brain regions that are well known to be rich in MOPr, such as the AcbSh, thalamus, hypothalamus, and amygdala, supporting the plausible existence of brain region-specific $mGlu_5R$-MOPr interactions; however, in the present study, direct receptor interactions have not been investigated. These brain regions are involved in the regulation of drug-induced reward (Bosser et al., 2007; Chaudhri et al., 2010) and withdrawal-induced emotional deficits (Zanos et al., 2014), as well as reinstatement (Georgiou et al., 2015). Our data add to the existing evidence for an association between the central $mGlu_5R$ and MOP systems (Schroder et al., 2009; Akgun et al., 2013; Smeester et al., 2014). Indirect evidence further suggests a possible modulatory inter-play between $mGlu_5R$ and MOPr systems in the striatum to regulate opioid-related behaviors, since intra-Acb administration of an $mGlu_5R$ antagonist attenuated the acquisition of morphine CPP (Roohi et al., 2014), and intra-AcbSh, but not intra-AcbC, administration of an $mGlu_5R$ antagonist attenuated both cue- and priming-induced reinstatement of heroin-seeking behavior in rats (Lou et al., 2014). The exact neurobiological mechanism underlying the observed $mGlu_5R$ upregulation in the MOPr knockout mice remains unclear, since glutamate content and/or release in the brain of MOPr knockout mice have not been evaluated so far. If glutamatergic neurotransmission in these mice is generally reduced, an upregulation of the $mGlu_5R$ may represent a compensatory mechanism to counteract the neuronal responses to decreased glutamate levels; however, in the present study we observed region-specific upregulation of $mGlu_5R$ in these mice and not a global effect, which indicates that the possibility of a direct molecular interaction between $mGlu_5R$ and MOPr in specific brain regions is more likely to exist. Indeed, GPCRs, including $mGlu_5R$ and MOPr, are capable of forming heterodimers, and it has been shown that this physical association can modulate receptor binding and function (Schroder et al., 2009; Smeester et al., 2014). If dimeric interactions exist between these 2 systems, the lack of MOPr may result in alterations of $mGlu_5R$ pharmacology in brain regions where the 2 receptors physically interact. Although this possibility is only speculative at present, it deserves further investigation.

Taken together, our findings showed that 6 to 8 days of morphine withdrawal induces emotional deficits in mice, which is concomitant with marked neuroadaptations in the $mGlu_5R$ system in the brain. These results suggest a possible involvement of the $mGlu_5R$ in the modulation of comorbid behavioral impairment during opioid abstinence and point toward targeting this system for the development of novel pharmacotherapies for opioid addiction. Moreover, our data suggest a direct inter-play between $mGlu_5R$ and MOPr in brain regions associated with drug addiction, reward, and emotions.

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Statement of Interest

None

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