# Loss of Morphine Reward and Dependence in Mice Lacking G Protein–Coupled Receptor Kinase 5

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**Background:** The clinical benefits of opioid drugs are counteracted by the development of tolerance and addiction. We provide in vivo evidence for the involvement of G protein–coupled receptor kinases (GRKs) in opioid dependence in addition to their roles in agonist-selective mu-opioid receptor (MOR) phosphorylation.

**Methods:** In vivo MOR phosphorylation was examined by immunoprecipitation and nanoflow liquid chromatography–tandem mass spectrometry analysis. Using the hot-plate and conditioned place preference test, we investigated opioid-related antinociception and reward effects in mice lacking GRK3 or GRK5.

**Results:** Etonitazene and fentanyl stimulated the in vivo phosphorylation of multiple carboxyl-terminal phosphate acceptor sites, including threonine 370, serine 375, and threonine 379, which was predominantly mediated by GRK3. By contrast, morphine promoted a selective phosphorylation of serine 375 that was predominantly mediated by GRK5. In contrast to GRK3 knockout mice, GRK5 knockout mice exhibited reduced antinociceptive responses after morphine administration and developed morphine tolerance similar to wild-type mice but fewer signs of physical dependence. Also, morphine was ineffective in inducing conditioned place preference in GRK5 knockout mice, whereas cocaine conditioned place preference was retained. However, the reward properties of morphine were evident in knock-in mice expressing a phosphorylation-deficient S375A mutation of the MOR.

**Conclusions:** These findings show for the first time that MOR phosphorylation is regulated by agonist-selective recruitment of distinct GRK isoforms that influence different opioid-related behaviors. Modulation of GRK5 function could serve as a new approach for preventing addiction to opioids, while maintaining the analgesic properties of opioid drugs at an effective level.

**Key Words:** Bar code, conditioned place preference, dependence, G protein–coupled receptor kinase, mu opioid receptor, phosphorylation

Although morphine is one of the most effective drugs for the treatment of severe pain, the clinical utility of morphine is limited by the rapid development of tolerance and the potential development of dependence and addiction after repeated or extended administration. Morphine exerts all of its pharmacologic effects through interactions with the mu-opioid receptor (MOR) (1–3). The efficiency of MOR signaling is tightly regulated and ultimately limited by the coordinated phosphorylation of intracellular serine and threonine residues. In HEK293 cells, agonist-induced phosphorylation of MORs occurs at a conserved 10-residue sequence, $^{370}$TREHPSTANT$^{379}$, in the carboxyl-terminal cytoplasmic tail (4,5). Morphine induces a selective phosphorylation of serine 375 (S375) in the middle of this sequence that is predominantly catalyzed by G protein–coupled receptor kinase 5 (GRK5) (6,7). As a consequence, the selective morphine-induced S375 phosphorylation does not lead to a robust beta-arrestin mobilization and receptor internalization (8). By contrast, high-efficacy opioid agonists such as DAMGO or etonitazene not only induce phosphorylation of S375 but also drive higher order phosphorylation on the flanking residues threonine 370 (T370), threonine 376 (T376), and threonine 379 (T379) in a hierarchical phosphorylation cascade that specifically requires GRK2 and GRK3 isoforms (5,6). As a consequence, multisite phosphorylation induced by potent agonist promotes both beta-arrestin mobilization and a robust receptor internalization (8). However, little is known about the physiologic consequences of these distinct opioid-induced MOR phosphorylation signatures.

Multiple isoforms of GRKs have been identified, but of the seven GRKs (GRK1 through GRK7), most receptors are potentially regulated by GRK2, GRK3, GRK5, or GRK6 (9). The GRK family is divided in three subgroups. One distinct subgroup that is found only in retinal cells comprises GRK1 (rhodopsin kinase) and GRK7 (cone opsin kinase). There are two subgroups within the non-visual GRKs: the GRK2 subfamily, consisting of GRK2 and GRK3, and the GRK4 subfamily, consisting of GRK4, GRK5, and GRK6. The GRKs GRK2, GRK3, GRK5, and GRK6 are widely expressed, especially in the brain (10–13), whereas GRK4 is predominantly found in the testes (14). The lack of GRK2 in mice is lethal in the embryo stage. Reduced antinociceptive tolerance to some opioid agonists is shown in GRK3 knockout mice (15,16). In contrast, GRK6 knockout mice do not exhibit altered analgesic responses to opioids (17).

In the mouse brain in vivo, only S375 phosphorylation has been demonstrated (18,19). Knock-in mice expressing a phosphorylation-deficient S375A mutant of MOR show enhanced analgesic responses and reduced tolerance to high-efficacy agonists but not to morphine, suggesting that disruption of MOR phosphorylation alters the behavioral effects of opioids (19). Whether the observed in vitro agonist-dependent hierarchical phosphorylation of MOR would occur in vivo and the identity of

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the GRKs that would participate in MOR phosphorylation remain unresolved. In the present study, by using mice lacking GRK3 or GRK5, we provide the first evidence that phosphorylation of endogenous MORs in the in vivo mouse brain is regulated by agonist-selective recruitment of distinct GRK isoforms. Such agonist-dependent GRK recruitment manifests into differential effects on several opioid-related behaviors independent of GRK-mediated phosphorylation of MOR.

## Methods and Materials

### Antibodies

The phosphorylation-independent rabbit monoclonal anti-MOR antibody (UMB-3) was obtained from Epitomics (Burlingame, California) (18). The guinea pig polyclonal phosphosite-specific antibody anti-pS375 (GM375-2) and the phosphorylation-independent guinea pig polyclonal anti-MOR antibody (GP6) were generated and extensively characterized in a previous study (18,19). The phosphosite-specific antibody for the T370-phosphorylated form of MOR (GM370-1) was generated against the IRQN(20)REHP sequence that contained a phosphorylated threonine residue and corresponded to amino acids 366–374 of the mouse MOR. The phosphosite-specific antibody for the T379-phosphorylated form of MOR (GM379-2) was generated against the STAN(20)VDRT sequence that contained a phosphorylated threonine residue and corresponded to amino acids 375–383 of the mouse MOR. The anti-pT370 guinea pig polyclonal antibody (GPM370-1) and the anti-pT379 guinea pig polyclonal antibody (GPM379-2) were generated and characterized in an identical manner as previously described for the anti-pT370 (3196) and anti-T379 (3686) rabbit polyclonal anti-MOR antibodies, respectively (4,5).

### Animals

Knock-in mice expressing the S375A mutant of the MOR ($Oprm1^{tm1Shlz}$) were generated and characterized as previously described (19). The GRK5 knock-out mice ($Grk5^{tm1Rjl}$) and GRK3 knock-out mice ($Adrbk2^{tm1Rjl}$) were obtained from Jackson Laboratory (Bar Harbor, Maine). Dr. H. Loh (University of Minnesota, Minneapolis, Minnesota) provided MOR knock-out ($^{-/-}$). Animals were housed under a 12-hour light-dark cycle with ad libitum access to food and water. All animal experiments were performed in accordance with the Thuringian state authorities and complied with European Commission regulations for the care and use of laboratory animals. Our study is reported in accordance with the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines for reporting experiments involving animals (21,22). For more information on drugs, behavioral test, in vivo MOR phosphorylation, and data analysis see Supplement 1.

## Results

### Hierarchical Multisite Phosphorylation of MORs In Vivo

To facilitate detection of multisite phosphorylated MORs in vivo, we first generated guinea pig polyclonal anti-pT370, anti-pS375, and anti-pT379 antibodies as well as the phosphorylation-independent rabbit monoclonal anti-MOR antibody (UMB-3). In UMB-3 immunoprecipitates from brain homogenates prepared from $MOR^{+/+}$ mice treated with the high-efficacy agonist etonitazene, we clearly detected phosphorylation of multiple sites, including T370, S375, and T379 (Figure 1A). By contrast, morphine stimulated phosphorylation at S375 but failed to stimulate robust phosphorylation at the other residues (Figure 1A and Figure S1A in Supplement 1), with equivalent receptor loading verified by detection of a distinct (nonphosphorylated) epitope in the cytoplasmic tail (Figure 1A, bottom panel). None of these bands were detected in brain homogenates prepared from $MOR^{-/-}$ mice after identical drug treatment (Figure 1A, Figure S1B in Supplement 1). Although weaker than that observed with etonitazene administration, fentanyl also promoted multisite phosphorylation of endogenous MORs (Figure 1B). As expected, when knock-in mice expressing an S375A mutation of the MOR ($MOR^{S375A/S375A}$) were treated with fentanyl, S375 phosphorylation was no longer detected (Figure 1B). Phosphorylation of T370 and T379 was also diminished in fentanyl-treated $MOR^{S375A/S375A}$ mice, although these residues were not mutated (Figure 1B, Figure S1C in Supplement 1). We used nanoflow liquid chromatography–tandem mass spectrometry analysis of UMB-3 immunoprecipitates to elucidate whether multiple phosphorylations occur in precisely the same receptor molecule, rather than being distributed over a mixture of singly phosphorylated receptor species. As depicted in Figure 1C, we detected an increase in receptor species with single phosphorylation at S375 and a marked increase in receptor species with double phosphorylation at S375 and T370 in response to etonitazene application in vivo. In contrast, the number of receptor species with a single phosphorylation at T370 remained unchanged (Figure 1C, Figure S2 in Supplement 1). Collectively, these results indicate that phosphorylation in this cytoplasmic region of MOR is hierarchical, with S375 representing an initiating site required for subsequent phosphorylation at T370 and T379 as reported earlier in HEK293 cells (5).

### Drug-Selective Engagement of Distinct GRK Isoforms In Vivo

In heterologous cells, ligand-induced MOR phosphorylation can be mediated by GRK2 and GRK3 as well as GRK5 isoforms (6). To evaluate the exact contribution of distinct GRK isoforms to MOR phosphorylation in vivo, we treated $GRK3^{-/-}$ and $GRK5^{-/-}$ mice with morphine or fentanyl and assessed S375 phosphorylation. Morphine-induced S375 phosphorylation was reduced in both $GRK3^{-/-}$ (~40%) and $GRK5^{-/-}$ (~50%) mice (Figure 1D,E). By contrast, fentanyl-induced S375 phosphorylation was reduced in $GRK3^{-/-}$ (~60%) but not in $GRK5^{-/-}$ mice (Figure 1F,G). Fentanyl-induced T370 phosphorylation was also reduced in $GRK3^{-/-}$ mice (~55%) (Figure S3 in Supplement 1). These findings suggest that GRK5 contributes selectively to morphine-induced S375 phosphorylation in vivo, whereas GRK3 is involved in both morphine-induced and fentanyl-induced S375 phosphorylation.

### Acute Antinociceptive Responses to Morphine Are Reduced in $GRK5^{-/-}$ Mice

With GRK5 being one of the GRKs involved, whether the phosphorylation of MOR at S375 would alter the agonist in vivo activity has not been addressed. After observing that $GRK5^{-/-}$ mice and wild-type (WT) littermates exhibited similar basal pain responses in the hot-plate test (not shown), we compared acute antinociceptive responses after subcutaneous administration of increasing doses of morphine (3–100 mg/kg). Under these conditions, $GRK5^{-/-}$ mice exhibited significantly weaker analgesic responses compared with WT mice [for genotype, $F_{1,31} = 30.17$, $p < .0001$; for dose, $F_{4,124} = 268.98$, $p < .0001$] (Figure 2A). In contrast, acute analgesic responses to increasing doses of fentanyl were not altered in $GRK5^{-/-}$ mice [for genotype, $F_{1,38} = .34$, $p = .5649$; for dose, $F_{3,114} = 912.29$, $p < .0001$] (Figure S4A in Supplement 1). To determine acute analgesic tolerance, $GRK5^{-/-}$ mice and their WT littermates were