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

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Abstract

Background—The clinical benefits of opioid drugs are counteracted by the development of tolerance and addiction. Here, 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 μ-opioid receptor phosphorylation.

Methods—In vivo μ-opioid receptor (MOR) phosphorylation was examined by immunoprecipitation and NanoLC-MS/MS analysis. Using the hot-plate and conditioned place preference (CPP) test we investigated opioid-related antinociception and reward effects in mice lacking GRK3 or GRK5.

Results—We show that etonitazene and fentanyl stimulate the *in vivo* phosphorylation of multiple carboxyl-terminal phosphate acceptor sites including threonine^{370} (T370), serine^{375} (S375), and threonine^{379} (T379), which is predominantly mediated by GRK3. By contrast, morphine promotes a selective phosphorylation of S375 that is predominantly mediated by GRK5. Unlike GRK3 knock-out mice, GRK5 knock-out mice exhibit reduced antinociceptive responses after morphine administration and develop morphine tolerance similar to wild-type mice but fewer signs of physical dependence. Also, morphine is not effective in inducing CPP in GRK5 knock-out mice, whereas cocaine CPP is retained. The rewarding properties of morphine, however, are evident in knock-in mice expressing a phosphorylation-deficient S375A mutation of the μ-opioid receptor.

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

Keywords

μ-opioid receptor; G protein-coupled receptor kinase; phosphorylation; conditioned place preference; dependence; bar code

Introduction

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 pharmacological effects through interactions with the 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 β-arrestin mobilization and receptor internalization (8). By contrast, high-efficacy opioids 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 GRK2/3 isoforms (5, 6). As a consequence, multi-site phosphorylation induced by potent agonist promotes both β-arrestin mobilization and a robust receptor internalization (8). However, little is known about the physiological consequences of these distinct opioid-induced MOR phosphorylation signatures.

Multiple isoforms of GRKs have been identified, but out of the seven GRKs (GRK1 through GRK7) most receptors are potentially regulated by only GRK2, GRK3, GRK5 or GRK6 (9). The GRK family is divided in three sub-groups whereas GRK1 (rhodopsin kinase) and GRK7 (cone opsin kinase) form one distinct sub-group that is only found in retinal cells. Within the non-visual GRKs there are two sub-groups: the GRK2 subfamily, consisting of GRK2 and GRK3, and the GRK4 subfamily, consisting of GRK4, GRK5 and GRK6. GRK 2, 3, 5 and 6 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 embryonic lethal, whereas GRK3 knock-out mice show reduced antinociceptive tolerance to some opioid agonists (15, 16). In contrast, GRK6 knock-out mice do not exhibit altered analgesic responses to opioids (17).

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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 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 will occur *in vivo* and the identity of the GRKs participate in such MOR phosphorylation remain unresolved. Here, 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.

Materials and Methods

Antibodies

The phosphorylation-independent rabbit monoclonal anti-MOR antibody (UMB-3) was obtained from Epitomics (Burlingame, CA) (18). The guinea pig polyclonal phosphosite-specific antibodies 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 to that 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). GRK5 knock-out mice (Grk5^{tm1Rjl}) and GRK3 knock-out mice (Adrbk2^{tm1Rjl}) were obtained from The Jackson Laboratory. MOR knock-out ($^{-/-}$) mice were provided by Dr. H. Loh (University of Minnesota, Minneapolis, MN). Animals were housed under a 12 h 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. Furthermore, our study is reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (21, 22). For more information on drugs, behavioral test, *in vivo* MOR phosphorylation and data analysis see supplemental information.

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Results

Hierarchical multi-site phosphorylation of MORs *in vivo*

To facilitate detection of multi-site 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, Supplemental Figure S1A), with equivalent receptor loading verified by detection of a distinct (non-phosphorylated) epitope in the cytoplasmic tail (Figure 1A, lowest panel). None of these bands were detected in brain homogenates prepared from $MOR^{-/-}$ mice after identical drug treatment (Figure 1A, Supplemental Figure S1B). Although weaker than those observed with etonitazene administration, fentanyl also promoted multi-site phosphorylation of endogenous MORs (Figure 1B). As expected, when knock-in mice expressing a S375A mutation of the μ-opioid receptor ($MOR^{S375A/S375A}$) were treated with fentanyl, S375 phosphorylation was no longer detected (Figure 1B). Remarkably, phosphorylation of T370 and T379 was also diminished in fentanyl-treated $MOR^{S375A/S375A}$ mice even though these residues were not mutated (Figure 1B, Supplemental Figure S1C). We then used NanoLC-MS/MS 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, Supplemental Figure S2). 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 GRK2/3 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 and 1E). By contrast, fentanyl-induced S375 phosphorylation was reduced only in $GRK3^{-/-}$ (~60%) but not in $GRK5^{-/-}$ mice (Figure 1F and 1G). Interestingly, fentanyl-induced T370 phosphorylation was also reduced in $GRK3^{-/-}$ mice (~55%) (Supplemental Figure S3). These findings suggest that GRK5 contributes selectively to morphine-induced S375 phosphorylation *in vivo*, whereas GRK3 is involved in both morphine- and fentanyl-induced S375 phosphorylation.

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Acute antinociceptive responses to morphine are reduced in $GRK5^{-/-}$ mice

With GRK5 being one of the GRKs involved, whether the phosphorylation of MOR at Ser375 will 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 (Figure 2A) (for genotype, $F (1,31) = 30.17$; $p <0.0001$; for dose, $F (4,124) = 268.98$; $p <0.0001$). In contrast, acute analgesic responses to increasing doses of fentanyl were not altered in $GRK5^{-/-}$ mice (Supplemental Figure S4A) (for genotype, $F (1,38) = 0.34$; $p = 0.5649$; for dose, $F (3,114) = 912.29$; $p <0.0001$). To determine acute analgesic tolerance, $GRK5^{-/-}$ mice and their WT littermates were challenged twice with morphine (10 mg/kg) and hot-plate latencies were measured. Again, morphine produced significantly less pronounced analgesia in $GRK5^{-/-}$ mice compared to WT mice (Supplemental Figure S5A). The antinociceptive effect of morphine persisted for approximately 240 min in both $GRK5^{-/-}$ and WT mice. A second morphine challenge 240 min after the first injection produced a strongly attenuated response, which was significantly different from the first response in both genotypes, demonstrating acute analgesic tolerance (Supplemental Figure S5A) (for genotype, $F (1,18) = 11.13$; $p = 0.0037$; for time, $F (4,72) = 202.47$; $p <0.0001$). These results suggest that GRK5-mediated S375 phosphorylation is not required for the development of acute analgesic tolerance after exposure to morphine. Next, chronic tolerance was induced by twice daily injections of morphine (10 mg/kg) for 10 consecutive days. Antinociceptive responses were measured in the hot-plate test 30 min after morphine administration on days 1, 3, 6, and 10. Again, $GRK5^{-/-}$ mice exhibited significantly weaker antinociceptive responses during the initial days of morphine administration (Figure 2B) (for genotype, $F (1,58)= 14.81$; $p = 0.0003$; for time $F (3,174) = 65.61$; $p <0.0001$). However, during chronic morphine administration, $GRK5^{-/-}$ and WT mice developed tolerance at a similar rate, with robust tolerance observed for both genotypes after 10 days (Figure 2B). Finally, withdrawal was elicited by injection of naloxone (2 mg/kg) 2 h after the last injection of morphine. As summarized in Figure 2C, $GRK5^{-/-}$ mice exhibited significantly fewer withdrawal symptoms than WT mice as assessed by number of jumps and wet-dog shakes (for jumps, mean ± SEM of $GRK5^{-/-}$ mice = 26.56 ± 2.042, of WT mice = 71.00 ± 3.194; for shakes, mean ± SEM of $GRK5^{-/-}$ mice = 3.778 ± 0.4120, of WT mice = 8.556 ± 0.4843). These findings suggest that $GRK5^{-/-}$ mice developed fewer signs of physical dependence but with similar degree of tolerance after morphine administration.

Under identical testing conditions, $GRK3^{-/-}$ mice exhibited acute antinociceptive responses to morphine (for genotype, $F (1,8) = 0.20$; $p = 0.6622$; for dose, $F (4,72) = 415.57$; $p < 0.0001$) and developed a tolerance to chronic morphine similar to that observed for WT mice (for genotype, $F (1,6) = 0.75$; $p = 0.3934$; for time, $F (3,108) = 38.30$; $p <0.0001$) (Figure 2D and 2E). In addition, after subcutaneous administration of increasing doses of fentanyl $GRK3^{-/-}$ mice exhibited acute analgesic responses similar to those observed for WT mice (Supplemental Figure S4B) (for genotype, $F (1,18) = 2.74$; $p = 0.1153$; for dose, $F (3,54) = 282.25$; $p <0.0001$). Consistent with previous reports, however, $GRK3^{-/-}$ mice showed less acute analgesic tolerance to the high-efficacy agonist fentanyl (15, 16). In fact, subcutaneous

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injection of 0.3 mg/kg fentanyl resulted in similar antinociceptive responses in GRK3$^{-/-}$ and WT mice, measured as hot-plate latencies. The analgesic effect of fentanyl lasted for approximately 180 min. 180 min after the first injection, a second fentanyl challenge provoked a similar analgesic response compared to the first in GRK3$^{-/-}$ mice ($p>0.05$; Supplemental Figure S5B), indicating that GRK3$^{-/-}$ mice do not develop acute analgesic tolerance to fentanyl. In contrast, WT mice exhibited significantly reduced analgesia after the second injection compared to the first (Supplemental Figure S5B) (for genotype, $F(1,18) = 3.53$; $p = 0.0767$; for time, $F(4,72) = 515.51$; $p < 0.0001$). Moreover, GRK3$^{-/-}$ mice showed significantly less tolerance to repeated administration of the long-acting, high-efficacy agonist etonitazene (Figure 2f) (for genotype, $F(1,8) = 1.43$; $p = 0.2479$; for time, $F(3,54) = 31.95$; $p < 0.0001$). These results suggest that GRK3-mediated multi-site phosphorylation facilitates the development of acute and chronic tolerance after exposure to high-efficacy agonists.

Loss of morphine conditioned place preference (CPP) in GRK5$^{-/-}$ mice

With a reduction in the somatic withdrawal signs in GRK5$^{-/-}$ mice after chronic morphine administration, it is likely that the motivational withdrawal related to the drug's rewarding property can also be reduced in these mice. Therefore, a CPP paradigm was used to evaluate whether the rewarding properties of morphine were affected by deletion of GRK5. Interestingly, after conditioning with morphine for 4 days, GRK5$^{-/-}$ mice did not show a preference for the drug-paired chamber but instead developed a strong aversion that persisted for 3 weeks (Figure 3A) (for genotype $F(1,18) = 168.06$; $p < 0.0001$; for time $F(5,90) = 4.36$; $p = 0.0013$). Similarly, conditioning with fentanyl also resulted in a place aversion that persisted for 2 weeks (Figure 3B) (for genotype, $F(1,18)= 42.22$; $p < 0.0001$; for time, $F(5,90) = 0.41$; $p = 0.8400$). The activation of both MORs and D_2 dopamine receptors plays a critical role in the reinforcing effects of morphine (1, 23). To test whether deletion of GRK5 alters dopaminergic signaling, GRK5$^{-/-}$ mice were conditioned with cocaine. Both the magnitude and extinction of cocaine CPP were similar in GRK5$^{-/-}$ and WT mice (Figure 3C) (for genotype, $F(1,10) = 0{,}40$; $p = 0.5436$; for time, $F(4,40) = 37{,}61$; $p < 0.0001$), suggesting that dopamine receptor function is not affected by elimination of GRK5. Similar experiments performed in GRK3-deficient mice show that GRK3$^{-/-}$ mice developed a strong preference for the drug-paired side after conditioning with morphine (for genotype, $F(1,18) = 4.44$; $p = 0.0494$; for time $F(5,90) = 37.35$; $p < 0.0001$) or fentanyl (for genotype, $F(1,17) = 4.58$; $p = 0.0472$; for time, $F(5,85) = 36.71$; $p < 0.0001$) (Figure 3D and 3E). However, GRK3$^{-/-}$ mice showed faster extinction of morphine and fentanyl CPP than WT mice (Figure 3D and 3E). In order to test the potential influence of the GRK3 deletion on dopaminergic signaling, GRK3$^{-/-}$ mice were conditioned with cocaine. After conditioning with cocaine for 4 days, GRK3$^{-/-}$ mice exhibited a preference for the drug-paired chamber. Moreover both the magnitude and extinction of cocaine CPP were similar in GRK3$^{-/-}$ and WT mice (Supplemental Figure S6) (for genotype, $F(1,10) = 0.18$; $p = 0.6839$; for time, $F(5,50) = 2.21$; $p = 0.0675$), suggesting that also elimination of GRK3 has no dramatic impact on dopamine receptor function.

As GRK5 is involved in the *in vivo* S375 phosphorylation of the morphine-activated MOR (Figure 1C), we next evaluated whether GRK5-mediated MOR phosphorylation is required

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for the reinforcing properties of morphine in phosphorylation-deficient $MOR^{S375A/S375A}$ mice. $MOR^{S375A/S375A}$ mice developed a strong preference for the drug-paired side (Figure 3F). However, similar to that observed for $GRK3^{-/-}$ mice, $MOR^{S375A/S375A}$ mice showed faster extinction of morphine CPP than WT mice (Figure 3F) (for genotype, $F (1,5) = 32.30$; $p <0.0001$; for time, $F (5,75) = 46.76$; $p <0.0001$). The CPP results suggest that functions of GRK5 other than mediating S375 phosphorylation of MOR are essential for morphine reward. In many receptor systems, siRNA knock-down of GRK5 inhibits agonist-driven extracellular-signal-regulated kinase (ERK) activation, suggesting that this pathway is a major downstream effector of GRK5 (24–27). If GRK5 signals through ERK during the development of morphine CPP *in vivo*, then chemical inhibition of this pathway should lead to a defect similar to that observed for $GRK5^{-/-}$ mice. Indeed, intraperitoneal injection of the MEK1/MEK2 inhibitor SL327 1 h before morphine administration completely blocked morphine CPP in WT mice (Figure 4A) (for genotype, $F (1,18) = 36.94$; $p <0.0001$; for time, $F (5,90) = 8.36$; $p <0.0001$). Pretreatment with SL327 did neither affect morphine analgesia (Figure 4B) (mean ± SEM = 54.36 ± 4.093 for mice injected with SL327; mean ± SEM = 56.64 ± 3.900 for mice injected with NaCl) nor the development of tolerance after chronic injection of 10 mg/kg morphine (Figure 4C). Either group, mice injected with NaCl and mice injected with SL327, exhibited tolerance at a similar rate, resulting in a robust tolerance after 10 days (for genotype, $F (1,18) = 0.05$; $p =0.8272$; for time, $F (3,54) = 243.36$; $p <0.0001$).

Discussion

For many G protein-coupled receptors (GPCRs), the agonist-driven phosphorylation of intracellular serine and threonine clusters directly regulates their affinity for β-arrestin. Accumulating evidence suggests that distinct GRKs are preferentially recruited to and phosphorylate receptors as a function of their ligand-induced conformation (24, 28, 29). In this manner, different GRKs act as sensors that detect active receptor conformations stabilized by different ligands. This preferential recruitment of GRKs leads to distinct phosphorylation patterns (or “bar codes”) in the carboxyl-terminal cytoplasmic tails, which regulate the nature of β-arrestin intracellular functions (24, 28, 29). However, until now, the bar code hypothesis has been solely based on evidence from *in vitro* studies examining site-specific phosphorylation and cellular functions of GRKs in heterologous expression systems (20, 30, 31).

Here, we demonstrate, for the first time, that different ligand-dependent phosphorylation bar codes can be established *in vivo* by drug-selective engagement of distinct GRK isoforms and we bring the proof of *in vivo* multiphosphorylation sites on a single MOR, by applying for the first time mass spectrometry to brain endogenous receptor. Furthermore, agonist-selective recruitment of GRKs influences different drug-related behaviors. High-efficacy agonists such as etonitazene and fentanyl stimulate the phosphorylation of multiple phosphate acceptor sites in a conserved 10-residue sequence, ^{370}TREHPSTANT^{379}, in the MOR cytoplasmic tail, including T370, S375, and T379. By contrast, low-efficacy agonists such as morphine stimulate robust phosphorylation of S375 only. The use of GRK-deficient mice allowed us to decipher the specific GRK isoforms involved in this process *in vivo*. Our results indicate that GRK5 selectively contributes to morphine-induced S375

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phosphorylation, whereas GRK3 is involved in both morphine- and fentanyl-induced S375 phosphorylation. These *in vivo* findings confirm our previous observations in transfected HEK293 cells (4–6). Furthermore, our current studies reveal an additional level of complexity, in that GRK2 and GRK3 can function as redundant phosphorylation system. Simultaneous knock-down of both GRK2 and GRK3 is required for the near-complete inhibition of MOR phosphorylation after activation by high-efficacy agonists (5, 6). The redundancy in the involvement of both GRK2 and GRK3 could explain our current observation that ~40% phosphorylation at S375 remained in GRK3$^{-/-}$ mice without the participation of GRK5 in the fentanyl-induced MOR phosphorylation at this Serine residue.

Earlier *in vitro* studies implicate GRK2/3 phosphorylation sites primarily in receptor desensitization and internalization, whereas GRK5/6 sites appear to be involved in β-arrestin-mediated ERK activation (31, 32). These findings may account for some of our behavioral results. That is, in the absence of GRK5, GRK2/3 may gain access to morphine-activated MORs and facilitate their desensitization simply by sequestering G proteins resulting in weaker antinociceptive responses after morphine administration in GRK5$^{-/-}$ mice. Alternatively, GRK5 may serve a scaffolding function that facilitates signaling through the morphine-activated MOR. However, this effect appears to be selective for MOR. The antinociceptive effects of oxotremorine are potentiated and prolonged in GRK5$^{-/-}$ mice, suggesting a role for GRK5 in muscarinic receptor desensitization (33). In addition, the loss of GRK3 may be partly compensated by GRK2, rendering the defect in MOR desensitization detectable only when MOR is activated repeatedly by high-efficacy agonists. Unlike GRK5$^{-/-}$ mice, both GRK3$^{-/-}$ and S375A knock-in mice developed a strong preference for the morphine-paired side in the CPP test, suggesting that GRK5-driven downstream signals rather than S375 phosphorylation of the receptor itself are critical for morphine CPP. Thus, inhibiting GRK5 function might serve as a new approach for eluding the rewarding effects of opioids while maintaining their analgesic properties at a still effective level.

Earlier studies have also revealed complex alterations in opioid-related behaviors in GRK6$^{-/-}$ mice. Acute morphine treatment induces greater locomotor activity but less constipation in these animals, however they display a similar CPP compared to WT. In addition, analgesic tolerance and physical dependence are not affected by ablation of the GRK6 gene, suggesting that this gene may only be responsible for a discrete subset of morphine-mediated responses (17). In contrast, mice lacking β-arrestin2 display enhanced and prolonged response latencies in the hot plate test upon challenge with morphine and do not develop morphine tolerance, which suggests a negative regulatory role for β-arrestin2 in desensitizing the morphine-bound MOR. Moreover, while mice lacking β-arrestin2 display enhanced antinociceptive responses to morphine, their responses to methadone, fentanyl and etorphine do not differ from WT mice (34–37). These studies contribute to the concept of "biased agonism", suggesting that different agonists at the MOR interact preferentially with certain cellular proteins to mediate distinct biological responses. Nevertheless, MORs expressed in different tissues and neuronal populations, or even neuron locations could be differentially sensitive to the regulation of kinases and β-arrestin2. This is best exemplified by the ability of morphine to induce MOR internalization within the dendritic process but

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not in soma of the nucleus accumbens neurons probably due to difference in β-arrestin levels (38).

Distinct agonist-induced MOR phosphorylation "bar codes" could have different physiological consequences. In $MOR^{S375A/S375A}$ mice, morphine and fentanyl provoked greater antinociceptive responses than in WT mice, whereas acute and chronic tolerance to morphine was retained. In contrast, antinociceptive tolerance after repeated subcutaneous application of etonitazene was diminished (19). These results suggest that tolerance to agonists with different efficacies develops through distinct pathways.

Morphine and cocaine, as well as many other major drugs of abuse, lead to an increase in dopamine signaling in mesolimbic brain structures such as the nucleus accumbens (39, 40). In the case of morphine, however, stimulation of dopamine pathways is indirect, originating from a disinhibition of GABAergic cells in the ventral tegmental area (41, 42). Unlike those observed with morphine, the rewarding properties of cocaine were retained in $GRK5^{-/-}$ mice, suggesting that the loss of morphine CPP does not result from an alteration of dopamine receptor function by ablation of GRK5. Reduced numbers of dendritic spines in hippocampal neurons and slower learning in the Morris water maze have recently been reported for $GRK5^{-/-}$ mice (43). Nevertheless, the fact that cocaine CPP was retained also indicates that the lack of morphine CPP does not simply result from general memory impairment in $GRK5^{-/-}$ mice. Rather, this finding implies that GRK5 is specifically required for the reinforcing properties of morphine and fentanyl. One possible explanation could be that GRK5-mediated MOR phosphorylation will increase the affinity for β-arrestin and thereby facilitate ERK activation. In fact, the effects of GRK5 deletion can be mimicked in part by chemical inhibition of ERK signaling. Systemic application of SL327 shortly before each conditioning session was sufficient to completely block morphine CPP in WT mice without affecting morphine analgesia. However, the effects of SL327 are not selective, as systemic application of SL327 also inhibits cocaine CPP (44).

Formation and extinction of certain behavioral patterns associated with rewarding drugs underlie different processes of learning and memory (45, 46). In this context activation of ERK seems to be important for long-term plasticity and memory (47, 48). An increase in ERK phosphorylation in different brain areas of mice was found after acute and chronic application of morphine (49, 50). In striatal wild-type neurons, ERK phosphorylation was increased after stimulation with fentanyl (51). In contrast, in neurons derived from $GRK3^{-/-}$ mice the fentanyl-induced activation of ERK was diminished. These results could account for the faster extinction of fentanyl CPP in $GRK3^{-/-}$ mice.

The κ-opioid receptor (52) has an inhibitory effect on the brain reward system. Using KOR agonists like dynorphin or U50,488H it is possible to provoke a conditioned place aversion (CPA) and to attenuate the rewarding effects of morphine in rodents (53–56). Given that morphine can exert some agonistic effects via KOR, it is possible that the rewarding MOR effects of morphine are lost after ablation of GRK5, whereas the aversive KOR effects are thereby unmasked in these mice.

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Together, our findings represent compelling evidence that MOR phosphorylation is regulated by drug-selective engagement of distinct GRKs *in vivo*. As predicted by the bar code hypothesis, different GRKs act as sensors that detect active receptor conformations stabilized by different ligands. The preferential recruitment of GRKs leads to distinct MOR phosphorylation patterns that, in turn, influence different opioid-related behaviors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CPA	conditioned place aversion
CPP	conditioned place preference
ERK	extracellular-signal-regulated kinase
GRK	G protein-coupled receptor kinase
HEK 293	human embryonic kidney 293 cells
KOR	κ-opioid receptor
MOR	μ-opioid receptor
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
WT	wild type

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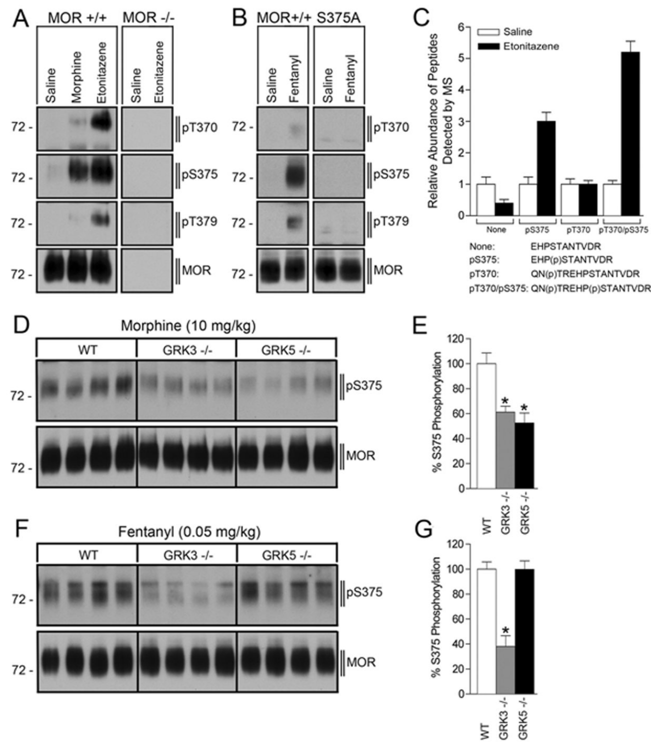

Figure 1. Agonist-selective MOR phosphorylation *in vivo*

(A) $MOR^{+/+}$ and $MOR^{-/-}$ mice were injected with saline, 30 mg/kg morphine, or 30 μg/kg etonitazene. (B) $MOR^{+/+}$ and $MOR^{S375A/S375A}$ mice were injected with saline or 0.3 mg/kg fentanyl. After 30 min, brains were dissected, and homogenates were prepared from the entire brain after removal of the cerebellum. MORs were immunoprecipitated with UMB-3 and immunoblotted with guinea pig anti-pT370 (first panel), anti-pS375 (second panel), or anti-pT379 (third panel) antibody. Blots were stripped and reprobed with phosphorylation-independent guinea pig anti-MOR antibody (fourth panel) to confirm equal loading of the gel. (C) MS analysis of immunoprecipitated MOP receptor from saline or etonitazene treated $MOR^{+/+}$ mice brain. After SDS-PAGE, trypsin digestion, and nanoLC-MS/MS decision tree-driven CID/ETD analyses, Mascot database searches, including putative phosphorylation modification, identified the MOP receptor sequence with 10–33% coverage, depending on the sample. Although limited, this coverage enabled us to unambiguously identify unphosphorylated and phosphorylated forms of T370 and S375 containing peptides. Phosphorylated and unphosphorylated peptides containing T370 and S375 were quantified using the MFPaQ software. For each peptide, results correspond to the MS peak intensity ratio of etonitazene-treated over saline control group and are expressed as mean ± SEM of n=3. (D) WT (n=4), $GRK3^{-/-}$ (n=4), and $GRK5^{-/-}$ (n=4) mice were injected with morphine. Brains were dissected after 30 min and processed as described above. (E) S375 phosphorylation was quantified and expressed as percentage of maximal phosphorylation in WT mice. Data are shown as mean ± standard error of the mean (SEM; n=10 per genotype). (F) WT (n=4), $GRK3^{-/-}$ (n=4), and $GRK5^{-/-}$ (n=4) mice were injected with fentanyl. Brains were dissected after 15 min and processed as described above. (G) S375 phosphorylation was quantified and expressed as percentage of maximal

phosphorylation in WT mice (n=10 per genotype). Positions of molecular mass markers (in kDa) are indicated to the left of the immunoblots. Data are presented as the means ± SEM. For all figures, *p<0.05.

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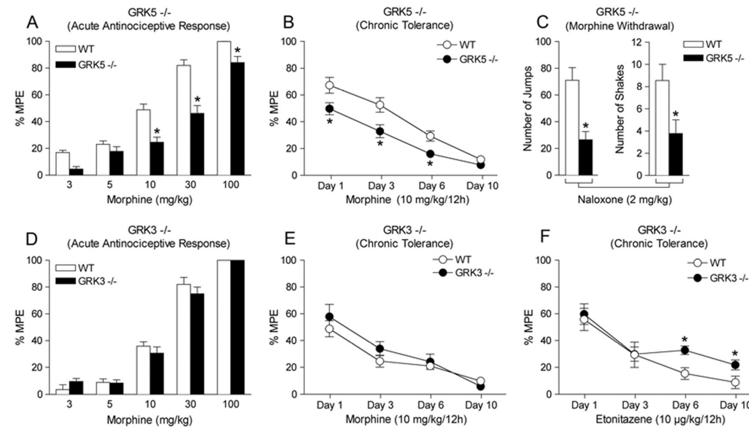

Figure 2. Reduced anti-nociceptive responses to morphine in GRK5$^{-/-}$ mice

(A) Pain responses in GRK5$^{-/-}$ and WT mice were measured in the hot-plate test. For morphine dose-response, hindpaw withdrawal latencies were measured 30 min after each of five injections of morphine: 3 mg/kg, 2 mg/kg (cumulative dose of 5 mg/kg), 5 mg/kg (cumulative dose of 10 mg/kg), 20 mg/kg (cumulative dose of 30 mg/kg), and 70 mg/kg (cumulative dose of 100 mg/kg). Data are shown as mean ± SEM of percentage maximum possible effect (% MPE) for WT (n=18) and GRK5$^{-/-}$ (n=15) mice (for genotype, F (1,31) = 30.17; p <0.0001; for dose, F (4,124) = 268.98; p <0.0001). (B) Tolerance in GRK5$^{-/-}$ (n=30) and WT (n=30) mice after twice daily injections of 10 mg/kg morphine for 10 days. Pain responses were measured 30 min after drug administration on days 1, 3, 6, and 10 (for genotype, F (1,58)= 14.81; p = 0.0003; for time F (3,174) = 65.61; p <0.0001). (C) After induction of chronic tolerance, withdrawal was precipitated by naloxone injection 2 h after the final morphine administration. Withdrawal symptoms were assessed by number of jumps and wet-dog shakes during a 30-min observation period (for jumps, mean ± SEM of GRK5$^{-/-}$ mice = 26.56 ± 2.042, of WT mice = 71.00 ± 3.194; for shakes, mean ± SEM of GRK5$^{-/-}$ mice = 3.778 ± 0.4120, of WT mice = 8.556 ± 0.4843; n = 9 per genotype). (D) Pain responses in GRK3$^{-/-}$ (n=10) and WT (n=10) mice (for genotype, F (1,18) = 0.20; p = 0.6622; for dose, F (4,72) = 415.57; p < 0.0001). (E) Tolerance to morphine in GRK3$^{-/-}$ (n=19) and WT (n=19) mice (for genotype, F (1,36) = 0.75; p = 0.3934; for time, F (3,108) = 38.30; p <0.0001). (F) Tolerance to etonitazene in GRK3$^{-/-}$ (n=10) and WT (n=10) mice (for genotype, F (1,8) = 1.43; p = 0.2479; for time, F (3,54) = 31.95; p <0.0001). All data are presented as the means ± SEM. For all figures, *p<0.05.

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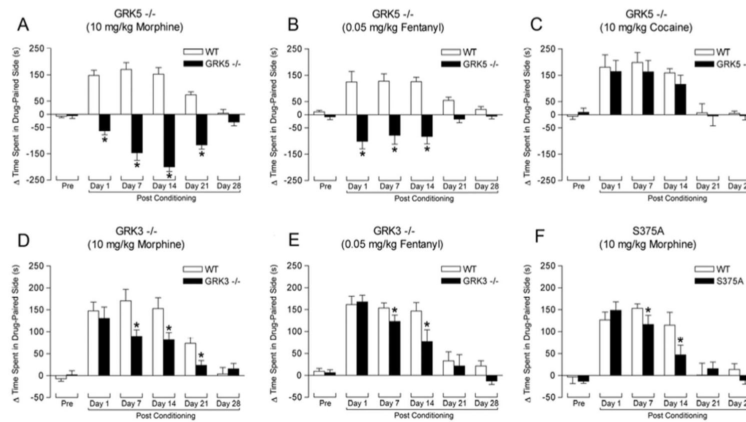

Figure 3. Loss of morphine-induced place preference in GRK5$^{-/-}$ mice

During the pre-conditioning phase (Pre), time spent in each side of the CPP apparatus was assessed. Drug was randomly paired with one side during conditioning. During the post-conditioning phase, time spent in each side was assessed on days 1, 7, 14, 21, and 28 in the absence of drug or saline. Data are shown as the difference in time spent in the drug-paired and saline-paired sides. (A) Morphine CPP in GRK5$^{-/-}$ (n=10) and WT (n=10) mice (for genotype $F(1,18) = 168.06$; $p < 0.0001$; for time $F(5,90) = 4.36$; $p = 0.0013$). (B) Fentanyl CPP in GRK5$^{-/-}$ (n=10) and WT (n=10) mice (for genotype, $F(1,18) = 42.22$; $p < 0.0001$; for time, $F(5,90) = 0.41$; $p = 0.8400$). (C) Cocaine CPP in GRK5$^{-/-}$ (n=6) and WT (n=6) mice (for genotype, $F(1,10) = 0,40$; $p = 0.5436$; for time, $F(4,40) = 37,61$; $p < 0.0001$). (D) Morphine CPP in GRK3$^{-/-}$ (n=10) and WT (n=10) mice (for genotype, $F(1,18) = 4.44$; $p = 0.0494$; for time $F(5,90) = 37.35$; $p < 0.0001$). (E) Fentanyl CPP in GRK3$^{-/-}$ (n=10) and WT (n=9) mice (for genotype, $F(1,17) = 4.58$; $p = 0.0472$; for time, $F(5,85) = 36.71$; $p < 0.0001$). (F) Morphine CPP in MOR$^{S375A/S375A}$ (n=8) and WT (n=9) mice (for genotype, $F(1,15) = 32.30$; $p < 0.0001$; for time, $F(5,75) = 46.76$; $p < 0.0001$). All data are presented as the means ± SEM. For all figures, $^*p<0.05$.

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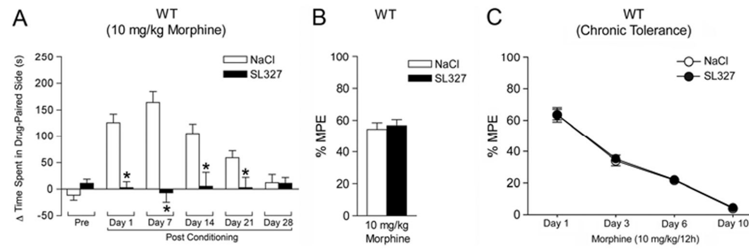

Figure 4. Inhibition of morphine-induced place preference by SL327

(A) Mice received saline (n=10) or MEK1/MEK2 inhibitor SL327 (10 mg/kg, n=10) 1 h before each morphine injection during CPP conditioning. During the post-conditioning phase, time spent in each side was assessed on days 1, 7, 14, 21, and 28 in the absence of drug or saline (for genotype, F (1,18) = 36.94; p <0.0001; for time, F (5,90) = 8.36; p <0.0001). (B) Pain responses of WT mice measured in the hot-plate test. Mice received saline (NaCl, n=8) or the MEK1/MEK2 inhibitor SL327 (10 mg/kg, n=10) 1 h before morphine injection. Hindpaw withdrawal latencies were measured 30 min after morphine injection (mean ± SEM = 54.36 ± 4.093 for mice injected with SL327; mean ± SEM = 56.64 ± 3.900 for mice injected with NaCl). (C) Effect of pretreatment with SL327 on the development of tolerance after twice daily injections of 10 mg/kg morphine. Pain responses of mice injected with NaCl (n=10) and mice injected with SL327 (10 mg/kg, n=10) 1 h before each morphine injection were measured 30 min after drug administration on days 1, 3, 6, and 10 (for genotype, F (1,18) = 0.05; p =0.8272; for time, F (3,54) = 243.36; p <0.0001). All data are presented as the means ± SEM. For all figures, *p<0.05.