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RGS7 regulates reward behavior by controlling opioid signaling in the striatum

Laurie P. Sutton[1], **Olga Ostrovskaya**[1], **Maria Dao**[2,3], **Keqiang Xie**[1], **Cesare Orlandi**[1], **Roy Smith**[3], **Sunmee Wee**[2], and **Kirill A. Martemyanov**[1,*]

[1]Department of Neuroscience, The Scripps Research Institute, Jupiter, FL 33458 USA

[2]Department of Molecular Therapeutics, The Scripps Research Institute, Jupiter, FL 33458 USA

[3]Department of Metabolism and Aging, The Scripps Research Institute, Jupiter, FL 33458 USA

Abstract

Background—Morphine mediates its euphoric and analgesic effects by acting on the μ-opioid receptor (MOR). MOR belongs to the family of G protein coupled receptors, whose signaling efficiency is controlled by the Regulator of G protein Signaling (RGS) proteins. Our understanding of the molecular diversity of RGS proteins that control MOR signaling, their circuit specific actions and underlying cellular mechanisms is very limited.

Method—We used genetic approaches to ablate RGS7 both globally and in specific neuronal populations. We used conditioned place preference and self-administration paradigms to examine reward-related behavior and a battery of tests to assess analgesia, tolerance and physical dependence to morphine. Electrophysiology approaches were applied to investigate the impact of RGS7 on morphine-induced alterations in neuronal excitability and plasticity of glutamatergic synapses. At least 3 animals were used for each assessment.

Results—Elimination of RGS7 enhanced reward, increased analgesia, delayed tolerance and heightened withdrawal in response to morphine administration. RGS7 in striatal neurons was selectively responsible for determining the sensitivity of rewarding and reinforcing behaviors to morphine, without affecting analgesia, tolerance and withdrawal. In contrast, deletion of RGS7 in dopaminergic neurons did not influence morphine reward. RGS7 exerted its effects by controlling morphine induced changes in excitability of medium spiny neurons in Nucleus Accumbens (NAc) and gating the compositional plasticity of AMPA and NMDA receptors.

Conclusion—This study identifies RGS7 as a novel regulator of MOR signaling by dissecting its circuit specific actions and pinpointing its role in regulating morphine reward by controlling the activity of NAc neurons.

*Corresponding author: Dr. Kirill Martemyanov, Department of Neuroscience, The Scripps Research Institute, 130 Scripps Way, 3C2, Jupiter, FL 33458, Phone: (561) 228-2770, kirill@scripps.edu.

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Keywords

G protein coupled receptors (GPCRs); opioids; striatum; reward circuit; Regulators of G protein Signaling (RGS); addiction

INTRODUCTION

The mesolimbic system is a major reward circuit in the brain consisting of projections from the dopaminergic neurons of the ventral tegmental area (VTA) to the nucleus accumbens (NAc) (1). The majority of the NAc is composed of medium spiny neurons (MSNs) that serve as the central output of the circuit (2, 3). As a result, changes in the activity of the MSNs have profound impact on reward behavior (4). The activity of MSNs is controlled by multiple neurotransmitters, many of which are acting on their cognate G protein coupled receptors (GPCRs), located on various neurons in the circuit. One such prominent system with a profound role in regulating the mesolimbic pathway is the endogenous opioid system, which underlies rewarding effects driving behavioral reinforcement for drugs of abuse such as opiates, alcohol and cannabinoids (5, 6). Pharmacological and genetic studies have shown that the central role in mediating the rewarding effects belongs to the μ-opioid receptor (MOR), which also serves as a direct target for abused opiates such as morphine and heroin (7–9).

The canonical model of reward and motivational action of opiates is described by a dopamine-dependent mechanism whereby activation of MOR on the GABAergic interneurons in the VTA leads to their diminished activity and results in the disinhibition of the dopaminergic VTA neurons facilitating the regulation of MSNs by dopamine (10, 11). MOR is also present on dopaminergic VTA neurons, directly modulating their activity and thus possibly contributing to dopamine-dependent effects of opioids (12, 13). However, strong evidence also points to significant role of dopamine-independent mechanisms in mediating morphine reward (14). Indeed, the MOR is prominently expressed directly on the MSNs in NAc (15) where it is involved in morphine reward-driven behaviors (16). Thus, a more encompassing model is now emerging that suggests that multiple synaptic mechanisms are likely contributing to the MOR-mediated reward behavior.

At the molecular level, strength and duration of GPCR signaling are potently regulated by members of the Regulator of G-protein Signaling (RGS) family (17, 18). RGS proteins act as GTPase-activating proteins (GAP) for G protein Gα subunit, thereby promoting their deactivation to result in faster termination of GPCR signaling. More than 30 individual RGS proteins are differentially expressed across the brain and several RGS proteins have been previously implicated in the overall regulation of opioid actions (19). Identifying which individual RGS proteins regulate MOR signaling within a specific neuronal population may help to distinguish their contributions *in vivo*. However, examples of the differential contribution to behavioral effects with the circuit level specificity of their action are limited. Here, we identify a novel role of RGS7 acting postsynaptically in MSNs of the NAc to specifically control rewarding and reinforcing effects of opioids.

MATERIAL AND METHODS

Animals and Viral Mediated Gene Transfer

All studies were carried out in accordance with the National Institute of Health guidelines and were granted formal approval by the Institutional Animal Care and Use Committee of the Scripps Research Institute. The generation of *Rgs7*$^{-/-}$ mice was described earlier (20). Conditional knockout mice were generated by crossing homozygous *Rgs7*$^{loxP/loxP}$ with heterozygous *DAT*^{cre} (B6.SJL-Slc6a3tm1.1(cre)Bkmn/J; Jackson Labs stock ID: 006660) or *Rgs9*^{cre} mice (21) to generate *Rgs7*$^{loxP/loxP}$*DAT*^{cre} and *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} knockout mice and their wildtype littermate controls, *Rgs7*$^{loxP/loxP}$ mice. The *Rgs9*^{cre} mice were bred with tdTomato reporter mice (B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG-tdTomato)Hze}$/J; Jackson Labs stock ID 007909). Mice were housed in groups on a 12 hr light–dark cycle with food and water available *ad libitum*. Males aged 2–5 months were used for all experiments except for the hotplate analgesia experiments where female mice were utilized. Details are provided in Supplement 1.

Western blotting and *In Situ* Hybridization

Western blotting was carried out according to published protocols (Supplement 1). For *In Situ* Hybridization ViewRNA^{TM} 2-plex *In Situ* Hybridization Assay (Panomics; Santa Clara, USA) was used (Supplement 1).

Behavioral Tests

Standard behavioral paradigms were applied to assess locomotor activity, drug induced reward, analgesic effects of acute and repeated morphine treatment and physical withdrawal. Full description of all behavioral tests can be found in Supplement 1.

Electrophysiology

Patch clamp recordings from ventral and dorsal striatal neurons in brain slices were used to measure neuronal excitability and determine AMPAR/NMDAR ratios in drug-naïve and morphine dependent mice. See Supplement 1 for a full description of protocols used.

Data Analysis

Statistical analysis was performed using GraphPad Prism (Prism 6.0, GraphPad, San Diego, CA). Groups were compared using one- or two-way ANOVA or Student's *t*-test where appropriate except for time-course experiments that used a repeated measures two-way ANOVA. Post hoc test consisted of Tukey's or Bonferroni as appropriate.

RESULTS

RGS7 regulates a broad spectrum of behavioral responses to morphine

We began by examining RGS7 expression in the mesolimbic system focusing on the VTA and the NAc (Fig. 1A, D). Using *in situ* hybridization technique at a single-cell resolution, we found an abundance of RGS7 mRNA in the majority of neurons in the VTA (Fig. 1B, C) and in the NAc (Fig. 1E, F). Co-labelling studies revealed that RGS7 and MOR were co-

expressed in the same neuronal populations in the VTA (Fig. 1B, C) and in the NAc (Fig. 1E, F).

To study the role of RGS7 in regulating opiate action with circuit-level resolution we generated several mouse lines using a conditional Cre-loxP strategy. We started with the phenotypic evaluation of mice with the brain-wide deletion of RGS7 (*Rgs7*$^{-/-}$) generated by crossing *Rgs7*$^{loxP/loxP}$ mice with a global germ-line EIIa^{Cre}-driver (Fig. 2A). Analysis of protein expression in the whole brain by western blotting, revealed no detectable RGS7 protein in the *Rgs7*$^{-/-}$ mice, indicating that RGS7 is completely eliminated using this strategy (Fig. 2B). This manipulation did not affect the expression of other closely related RGS proteins RGS9-2 and RGS6 but significantly reduced levels of RGS7 subunits: Gβ5 and R7BP (Fig. 2B).

To understand the overall contribution of RGS7 to opiate actions we performed behavioral characterization of *Rgs7*$^{-/-}$ mice. In the open filed, both *Rgs7*$^{-/-}$ mice and their wild-type littermates (*Rgs7*$^{+/+}$) displayed similar habituation to novel environment (Fig. 2C). However, *Rgs7*$^{-/-}$ mice demonstrated significantly lower levels of spontaneous locomotor activity during initial exploratory phase compared with *Rgs7*$^{+/+}$ mice (Fig. 2C) resulting in overall hypoactivity (Fig. 2D). Next, we examined the psychomotor activation of mice in response to morphine administration. *Rgs7*$^{-/-}$ mice showed increased sensitivity to morphine-induced locomotion, as these mutant mice showed enhanced locomotor activity at a lower dose than *Rgs7*$^{+/+}$ mice (Fig. 2E). At a dose that caused increased locomotor activity in both genotypes (10 mg/kg), morphine induced a maximal response at the same time (30 min), and the effect lasted for the same duration (~160 min) in both genotypes (Fig. 2F). However, locomotor response in *Rgs7*$^{-/-}$ mice was enhanced as compared to *Rgs7*$^{+/+}$ mice.

To assess possible changes in the rewarding effects of morphine, we used a place preference paradigm where mice are conditioned to prefer a drug-paired environment. Lack of RGS7 increased the sensitivity to morphine reward as *Rgs7*$^{-/-}$ mice exhibited a significant place preference at 1 mg/kg that was not observed in *Rgs7*$^{+/+}$ mice (Fig. 2G). As expected, a higher dose of morphine (10 mg/kg) induced comparable place preference in both *Rgs7*$^{+/+}$ and *Rgs7*$^{-/-}$ mice. No difference between genotypes in distance traveled during the test period was observed at any of the doses of morphine used (Fig. 2H).

We next examined responses of mutant mice to morphine analgesia. In a hot plate test, *Rgs7−/−* and *Rgs7*$^{+/+}$ mice exhibited comparable nociception threshold at a temperature of either 52°C or 56°C (Fig 3A). In contrast, following morphine treatment (5 mg/kg and 15 mg/kg) *Rgs7*$^{-/-}$ mice showed a substantially enhanced analgesic response compared to its *Rgs7*$^{+/+}$ controls (Fig. 3B). Pretreatment with naloxone, a MOR antagonist eliminated the analgesic effect of morphine in both genotypes. Furthermore, the duration of analgesic response to morphine (15 mg/kg) lasted longer in *Rgs7*$^{-/-}$ mice (Fig. 3C) as evidence by a significant decrease in morphine effectiveness at 90 min in *Rgs7*$^{+/+}$ mice and 150 min in *Rgs7*$^{-/-}$ mice.

Repeated opiate use leads to tolerance and a physical dependence that manifests in withdrawal and drug craving. Tolerance was measured by monitoring the morphine response

on the hotplate paradigm daily for 5 consecutive days. *Rgs7*$^{-/-}$ mice displayed a delayed tolerance to morphine compared with *Rgs7*$^{+/+}$ mice over the time course but both genotypes developed tolerance to morphine (Fig. 3D). We further examined the dependence of animals on morphine during a chronic administration regimen by scoring symptoms of physical withdrawal upon injection of an opioid receptor antagonist, naloxone. *Rgs7*$^{-/-}$ mice display more severe withdrawal symptoms as weight loss, diarrhea, wet dog shakes, tremors and ptosis are significantly increased compared to *Rgs7*$^{+/+}$ mice (Fig. 3E). Overall, these data show that mice lacking RGS7 exhibit enhanced rewarding and analgesic effects to morphine, greater dependence and delayed tolerance, thus demonstrating an essential role for RGS7 in opiate action.

RGS7 in dopaminergic neurons does not influence morphine reward

In order to determine the neuronal population responsible for the effect of RGS7 on morphine reward, we first tested the behavioral consequences of RGS7 elimination in dopamine neurons, a major neural substrate for the rewarding actions of opiates. We crossed the *Rgs7*$^{loxP/loxP}$ mice with *DAT*^{cre} driver line that expresses Cre recombinase in dopaminergic neurons (Figure S1A,B in Supplement) and compared the resulting *Rgs7*$^{loxP/loxP}$*DAT*^{cre} mice to their littermate controls *Rgs7*$^{loxP/loxP}$. In naïve mice, *Rgs7*$^{loxP/loxP}$*DAT*^{cre} and *Rgs7*$^{loxP/loxP}$ had similar locomotor activity habituation pattern in the open field (Figure S1C in Supplement). We further observed no differences in psychomotor activation between genotypes in response to morphine (Figure S1D in Supplement). Both genotypes showed an increase in locomotor activity to 10 mg/kg and 20 mg/kg morphine injections as compared to saline. Finally, we examined rewarding effects of morphine in the place preference paradigm. Both *Rgs7*$^{loxP/loxP}$*DAT*^{cre} and *Rgs7*$^{loxP/loxP}$ showed a significant place preference at 10 mg/kg but not at 1 mg/kg (Figure S1E in Supplement). No significant difference between the genotypes was observed for either morphine dose. These results indicate that dopaminergic neurons are not responsible for the effect of RGS7 on morphine-driven reward or psychomotor activation.

RGS7 in striatal neurons regulates morphine reward and reinforcement but not psychomotor activation, tolerance or withdrawal

To explore the possibility that post-synaptic RGS7 in the mesolimbic pathway is responsible for morphine-reward driven behaviors, we targeted RGS7 in striatal neurons. This was achieved by crossing the *Rgs7*$^{loxP/loxP}$ mice with the striatal specific driver line *Rgs9*^{cre}, where expression of Cre recombinase is restricted to post-synaptic neurons in the striatum (Fig. 4A,B; Figure S2 in Supplement). Evaluation of the resulting *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} mice in the open field under drug-naïve conditions revealed no difference compared with their *Rgs7*$^{loxP/loxP}$ controls (Fig. 4C). Administration of morphine resulted in significant increases in locomotor activity in both genotypes at both 10 mg/kg and 20 mg/kg with no difference between the genotypes (Fig. 4D). Next we investigated the rewarding effects of morphine in a CPP paradigm and found that only *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} mice showed a significant place preference towards the drug paired side at a low dose (1 mg/kg) of morphine (Fig. 4E). At a 10 mg/kg dose both *Rgs7*$^{loxP/loxP}$ and *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} exhibited a significant increase in time spent in the drug-paired side. At this higher dose, *Rgs7*$^{loxP/lox}$*Rgs9*^{cre} mice again showed an increased preference for the morphine-associated chamber as compared to

Rgs7$^{loxP/loxP}$ littermates. Next, we determined the contribution of ventral striatum in the actions of RGS7 by viral approach. Delivering AAV virus expressing Cre recombinase, but not control GFP expressing AAV, to the NAc significantly enhanced the of *Rgs7*$^{loxP/loxP}$ mice for the morphine-paired chamber at 1 mg/kg (Fig. 4F). Overall, the CPP data shows the involvement of RGS7 in the NAc in the rewarding properties of morphine.

We further performed a series of behavioral assays evaluating analgesia, morphine tolerance and withdrawal because the neuronal population where *RGS9*^{cre} driver is active has been previously shown to contribute to these behaviors (22). *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} and *Rgs7* $^{loxP/loxP}$ mice did not show any difference in nociceptive threshold in the hot plate test at either 52°C or 56°C (Fig. 4G). Furthermore, the analgesic responses to acute morphine administration were similar in both genotypes (Fig. 4H). Both *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} and *Rgs7*$^{loxP/loxP}$ mice also developed similar tolerance to daily injections of 15 mg/kg morphine on day 3 (Fig. 4I). Neither did we find any differences in withdrawal upon naloxone-precipitated morphine withdrawal (Fig. 4J). These findings suggest that rewarding properties of morphine are selectively affected by eliminating RGS7 in the striatum.

To confirm this conclusion we investigated the reinforcing properties of morphine using a morphine self-administration procedure which models drug reinforcing responses such as drug seeking and taking behavior. First, *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} mice and their littermate controls were trained in a two-lever operant task to respond to food reward (Fig. 5A). While both genotypes learned food self-administration to the set criteria, the *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} mice established this behavior earlier than their wild type littermates. Once responding for food reinforcement was established, mice were implanted with intravenous jugular catheters and assessed for lever pressing behavior that resulted in morphine infusions. Although both genotypes readily self-administered morphine at dose of 0.3 mg/kg/infusion, *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} mice exhibited an increased number of active lever presses compared with *Rgs7*$^{loxP/loxP}$ mice with no change on inactive lever pressing (Fig. 5B). This experiment demonstrates that *Rgs7* $^{loxP/loxP}$*Rgs9*^{cre} mice have a greater sensitivity to the reinforcing effects of morphine. Analysis of the dose-response relationship revealed that *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} mice earn a greater number of morphine infusions compared to *Rgs7* $^{loxP/loxP}$ mice at doses of 0.3 and 0.6 mg/kg/infusion (Fig. 5C). Furthermore, while both genotypes responded with increased morphine intake at higher doses (mg/kg/session), there was still a significant genotype difference (Fig. 5D). This upward shift of the dose-response relationship is thought to reflect the reinforcing effects of consumed morphine (23), supporting the hypothesis that deletion of RGS7 in striatum potentiates the rewarding effects of morphine thereby leading to an increase in morphine infusions.

RGS7 gates morphine-induced changes in intrinsic excitability and synaptic properties of striatal neurons

To determine the mechanisms by which striatal RGS7 regulates the rewarding effects of morphine we examined changes in the biophysical properties of MSN neurons in the NAc by whole cell patch clamp recordings. In drug naïve mice, we found no difference in several measures of intrinsic neuronal properties (Fig. 6A). In the basal state, NAc neurons of *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} mice and their *Rgs7*$^{loxP/loxP}$ littermates exhibited similar resting

membrane potentials (RMPs), input resistance and firing patterns in response to current injection (Fig. 6A, C–E). Next, we compared the same neuronal properties in mice that established stable morphine intake in self-administration paradigm one day following the last reinforced session. In contrast to naïve subjects, intrinsic excitability of NAc neurons was significantly diminished in self-administering $Rgs7^{loxP/loxP}Rgs9^{cre}$ mice relative to their control littermates as evident from the reduction in the number of action potentials (Fig. 6B) and elevation in the firing threshold (Fig. 6C). There was no difference in the RMP or input resistance across the genotypes (Fig. 6D,E). Similar changes in electrical properties were observed when morphine injections (10 mg/kg for 7 days) were delivered by an investigator (Figure S3 in Supplement), indicating that differences in neuronal excitability resulted from morphine effects rather than circuit entrainment associated with an operant behavior. Interestingly, these alterations were specific to NAc and no changes in the properties of neurons in the dorsal striatum were seen (Figure S4 in Supplement). Overall, these data suggest that differences in MSN excitability between $Rgs7^{loxP/loxP}$ and $Rgs7^{loxP/loxP}Rgs9^{cre}$ mice emerged in response to morphine administration.

In addition to regulating excitability, drugs of abuse are known to influence synaptic properties of neurons in the reward circuit, thus contributing to long-term plasticity that ultimately underlies drug-taking behavior. Thus, we next examined the role of RGS7 in regulating the composition of postsynaptic glutamate receptors, a well-known form of synaptic plasticity at glutamatergic synapses, influenced by addictive drugs and reward states (24). Under basal, drug naïve conditions afferent stimulation evoked comparable EPSCs in both $Rgs7^{loxP/loxP}$ and $Rgs7^{loxP/loxP}Rgs9^{cre}$ neurons in NAc (Fig. 7A). Dissection of individual receptor components using pharmacological blockade strategy revealed that the response is mediated by approximately equal contributions of AMPA and NMDA receptors with no difference in AMPAR/NMDAR ratio between genotypes (0.96 ± 0.14, n = 7 for $Rgs7^{loxP/loxP}$ and 0.93 ± 0.13, n = 7 for $Rgs7^{loxP/loxP}Rgs9^{cre}$). However, when measured in animals self-administering 0.3 mg/kg/infusion morphine, we detected a significant difference between the groups. $Rgs7^{loxP/lox}Rgs9^{cre}$ mice showed a significant increase in AMPAR/NMDAR ratio (1.5 ± 0.2, n = 6) as compared to $Rgs7^{loxP/loxP}$ littermates (1.1 ± 0.1, n = 9) when recorded one day following the last reinforced session (Fig 7B). Consistent with the post-synaptic nature of the manipulation with RGS7, we observed no change in the short-term pre-synaptic plasticity as evidenced examining paired pulse ratio (Fig 7C). Overall, these results suggest that loss of post-synaptic RGS7 selectively influences morphine-induced adaptation of intrinsic properties and glutamatergic receptor plasticity in NAc neurons.

DISCUSSION

This study demonstrates that RGS7 acts as a negative regulator of acute and chronic morphine actions. The behavioral experiments show that the lack of RGS7 results in a greater sensitivity to the rewarding and analgesic effects of morphine, as well as an increased somatic withdrawal response. Our data further dissociate the contribution of RGS7 within the neurons of the mesolimbic circuit specifically for reward and motivation. Using a conditional-knockout strategy, we report that RGS7 in the striatal MSNs but not in dopaminergic neurons of the midbrain regulates rewarding effects of morphine. We further

found that RGS7 was responsible for regulating morphine-induced glutamate receptor plasticity in NAc neurons and that adaptation of their intrinsic excitability in response to morphine drives an increased motivation of mice to engage in drug-taking behavior.

RGS7 is a member of the R7 family of RGS proteins, which negatively regulates GPCR signaling via inhibitory G proteins of the Gi/o class. As a classical Gi/o-coupled receptor, MOR is expected to be influenced by RGS7 activity. Indeed, our direct biochemical measurements indicate that RGS7 can potently regulate MOR signaling to Gαi and Gαo in reconstituted cellular system (25). Thus, enhanced morphine effects seen in knockout mice reported in this study, as well as earlier observations that antisense knockdown of RGS7 in the mouse brain enhanced morphine analgesia (26) are consistent with the mechanism that RGS7 acts by attenuating MOR signaling via Gαi/o.

The results of the present study reveal an underappreciated complexity of MOR regulation *in vivo*, as it is becoming increasingly apparent that the function of this receptor is differentially shaped by several RGS proteins across different brain regions. In addition to RGS7, depletion of RGS4 and RGS9 in the NAc also lowers the threshold for the rewarding effects of morphine producing a similar leftward shift in the CPP paradigm (27–29). Despite the diversity of RGS proteins regulating MOR in the NAc, all three RGS proteins appear to play non-redundant function as loss of one cannot be compensated by others. Furthermore, elimination of individual RGS in NAc neurons produce distinct phenotypes. For example, elimination of RGS9-2, also results in a greater analgesia, delayed tolerance and more severe somatic withdrawal symptoms (22). The genetic strategy that we used in this study afforded a unique opportunity to compare related RGS proteins, RGS7 and RGS9-2 in the regulation of morphine responses in the same neuronal populations. Strikingly, elimination of RGS7 in the same NAc neurons selectively affects reward with no impact on nociception, tolerance or withdrawal, despite the observations that globally RGS7 is involved in controlling all of these behaviors. The observation that RGS7 and RGS9-2 while acting in the same neurons produce distinct behavioral profiles suggests the existence of mechanisms that specify RGS action on MOR at the molecular level. While more work needs to be done to clarify such mechanisms, the realm of possibilities includes differential interactions of RGS proteins with their auxiliary regulatory subunits (30) and/or assembly of selective complexes with the G protein subunits (31). These mechanisms may work separately or together to bias different signaling outcomes downstream from MOR activation.

Using an operant self-administration task we determined that RGS7 affects the rewarding aspects of drug taking and seeking behaviors as animals lacking RGS7 showed increased morphine intake. In addition, when tested in the food self-administration paradigm mice lacking RGS7 in the striatum responded to the food reward earlier than their wildtype littermates suggesting that RGS7 may have a role beyond morphine-reward responses. The opioid system plays a fundamental role in the hedonic responses to natural rewards as well as in the learning processes that mediate goal-directed actions (32). We do not think that accelerated acquisition of operant responses to food upon deletion of RGS7 is associated with enhanced learning, as we previously found that $Rgs7^{-/-}$ mice exhibit deficits in learning and memory (33). Therefore, it is tempting to speculate that by controlling

endogenous opioid signaling through MOR, striatal RGS7 may be also involved in regulating hedonic properties of natural rewards.

One of the major observations of this work is that RGS7 controls morphine induced adaptation in the activity and synaptic plasticity of NAc neurons. Drugs of abuse, including morphine are well known to produce a variety of changes including structural alterations in the morphology of dendritic spines, as well as functional adaptation of firing properties and synaptic plasticity (34). It is thought that collectively these neuroadaptive changes underlie the induction of the lasting modifications of the mesolimbic circuit responsible for rewarding and reinforcing effects of drugs that contribute to the development of drug addiction (35). One prominent form of such adaptive effects triggered by opioids in the striatum is modulation of glutamatergic neurotransmission (36). Ample evidence points to the extensive cross-talk between the opioid and glutamate systems through several mechanisms that include modulation of glutamate release (37, 38) and composition of postsynaptic AMPA and NMDA glutamate receptors (39). The common theme behind these alterations seems to be an increase in the synaptic strength of NAc neurons that occurs as a result of either potentiating AMPA or inhibiting NMDA receptors. This is typically reflected in the increase in the AMPA/NMDA receptor ratio, a commonly used measure of the synaptic grading (40). Changes in AMPAR/NMDAR ratio is a hallmark adaptation triggered by many addictive drugs linked to increased motivated drug seeking behavior or relapse (24, 41). However, the mechanisms and molecular players that control the induction of this form of the synaptic plasticity are largely unknown. In this context, our observation that elimination of RGS7 specifically promotes the increase in AMPAR/NMDAR ratio induced by chronic morphine-taking behavior with no influence seen in the drug-naïve mice indicates that RGS7 gates the induction of this form of the synaptic plasticity. It is likely that RGS7 does so by controlling the extent of the G protein signaling by MOR, which leads to either enhanced inhibition of NMDA receptors or increased activity through AMPA receptors. Regardless of the exact mechanism, the use of selective genetic drivers allowed us to conclude that RGS7 acts directly in the postsynaptic striatal neurons in a cell autonomous manner without affecting the major dopaminergic input.

Another major adaptation caused by opioids is changes in neuronal excitability (42). Indeed, repeated morphine exposure induce a decrease in the intrinsic excitability of NAc neurons (43), while long-term withdrawal from repeated morphine elicits an increase intrinsic excitability of MSNs (44, 45). Consistent with these effects and the role of RGS7 in regulating the extent of G protein signaling by MOR, we find that elimination of RGS7 leads to a pronounced decrease in intrinsic excitability of NAc neurons in response to chronic morphine administration. Again, this effect was specifically induced by chronic morphine administration and not observed in naïve mice. While opioids appear to affect both intrinsic excitability and synaptic plasticity, it is not well understood how these effects are linked. Generally, molecular elements that affect excitability are located away from the synapse (e.g. voltage-gated ion channels and inward rectifiers), while AMPA and NMDA receptors adjust synaptic properties. Thus, RGS7 may contribute to regulation of two molecularly distinct processes associated with the activation of MOR receptors.

Taken together, we show that RGS7 is responsible for the neuronal adaptation associated with rewarding and reinforcing effects of morphine by regulating intrinsic excitability and plasticity of synaptic glutamate receptors. These findings may provide a better understanding of the molecular mechanism involved in diverse actions of morphine use by dissociating its circuit specific action.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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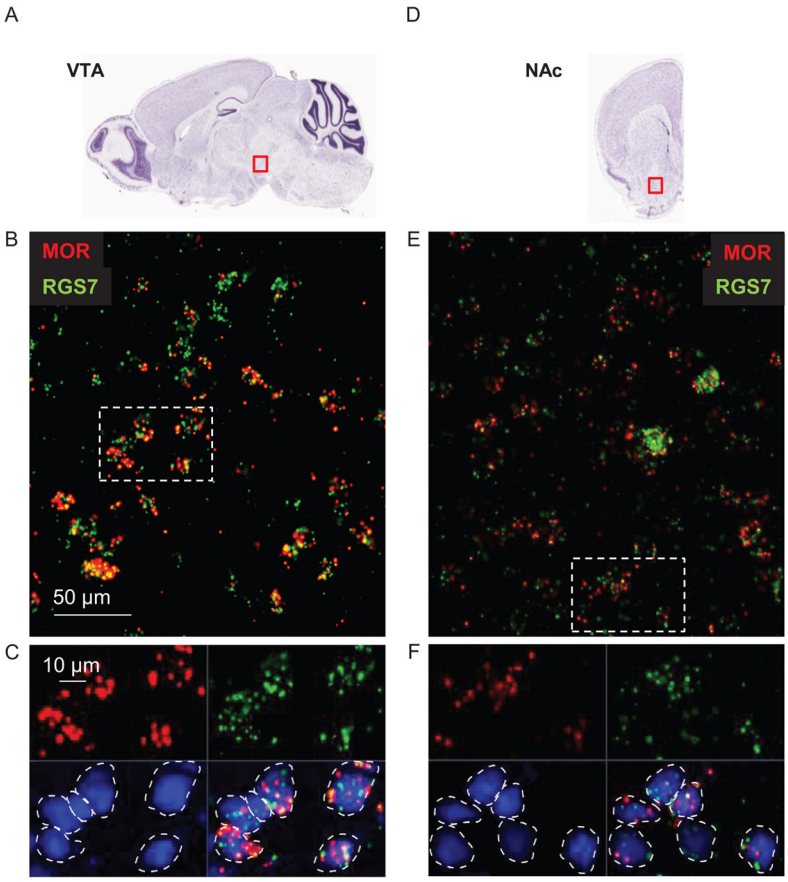

Figure 1. mRNAs for RGS7 and MOR are extensively co-expressed by neurons in the VTA and NAc

(A) Scheme representing a sagittal section of a mouse brain. The red square identifies the region (VTA) used for imaging. (B) Representative image of a double *in situ* hybridization using probes against MOR (red) and RGS7 (green) in the VTA. The dashed line defines the area shown at a higher magnification in panel C. (C) The red channel reports MOR transcripts encoding while the green channel reports localization of RGS7 mRNAs. The soma of each cell is identified by Nissl staining (blue) and its boundaries are delimited with a dashed line used to assign mRNA expression to individual neurons. (D) Scheme of a coronal section of a mouse brain. The red square identifies the region (NAc) used for imaging. (E) Double *in situ* hybridization of the MOR mRNA (red) and RGS7 mRNA (green) in the NAc. The dashed line defines the area reported at a higher magnification in panel F. (F) MOR mRNA (red) and RGS7 mRNA (green) co-expression in the NAc. The area of each soma is defined by Nissl staining (blue) and outlined by a dashed line. *In situ* hybridizations were conducted with sections from 3 separate mice. Representative images are shown.

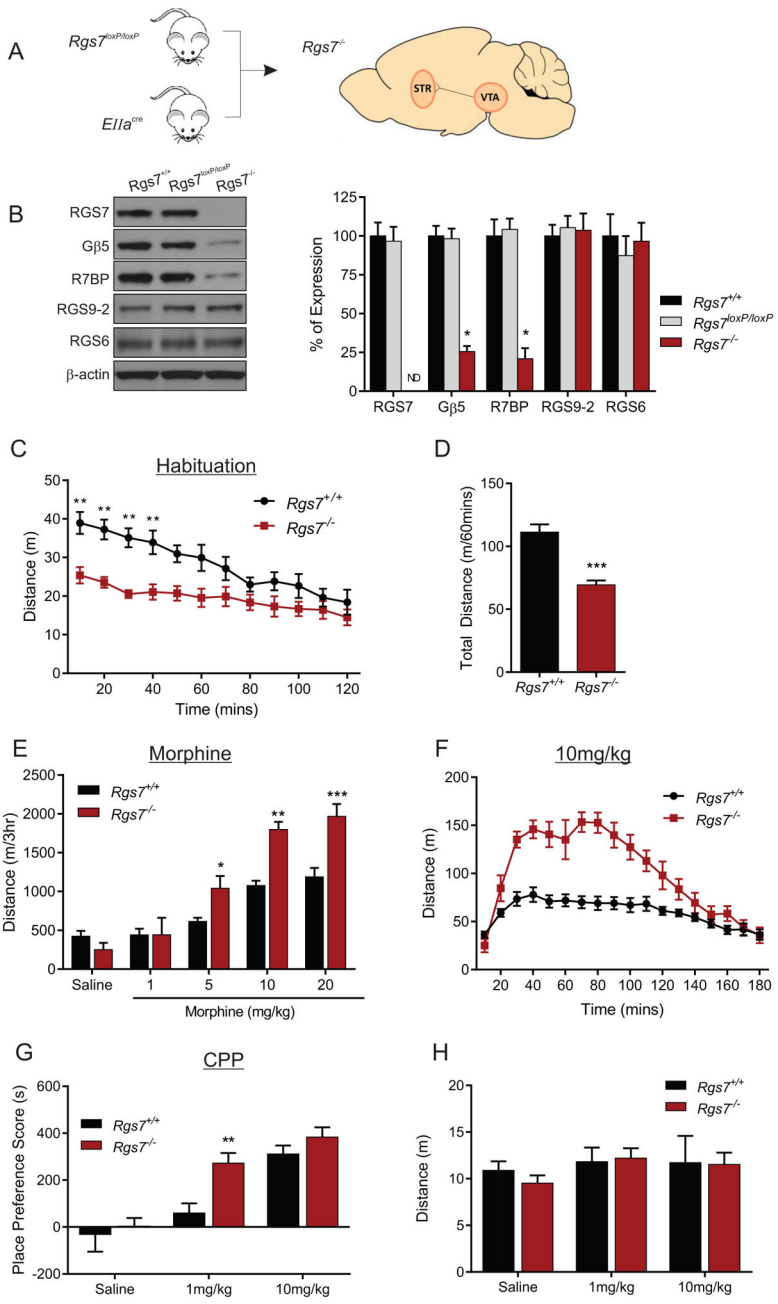

Figure 2. Global knockout of RGS7 increases sensitivity to morphine-induced locomotion and conditioned place preference (CPP)

(A) Schematic for the generation of *Rgs7*$^{-/-}$ mice. (B) Western blot analysis of protein expression in the whole brain (n = 6 per group, compared to *Rgs7*$^{+/+}$). (C) Locomotor activity in 10 min bins (genotype $F_{(11, 156)} = 6.993$, $p < 0.0001$; time $F_{(1, 156)} = 66.77$, $p < 0.0001$) or (D) cumulative distance during habituation show hypoactivity in *Rgs7*$^{-/-}$ mice compared with *Rgs7*$^{+/+}$ (n = 6–9 per group). (E) Locomotor distance for mice treated with varying doses of morphine (n = 6–9 per treatment; treatment $F_{(1, 65)} = 25.49$, $p < 0.0001$; dose $F_{(4, 65)} = 52.64$ and interaction $F_{(4, 65)} = 7.471$, $p < 0.0001$). (F) Time course of

distance traveled for mice injected with 10 mg/kg morphine (genotype $F_{(1, 234)} = 214.7$, $p < 0.0001$; time $F_{(17, 234)} = 23.2$, $p < 0.0001$ and interaction $F_{(17, 234)} = 6.994$, $p < 0.0001$). (G) Effects of morphine-induced CPP at doses of 1 mg/kg and 10 mg/kg (n = 6–11/group. Place preference scores are calculated as the difference between time spent in the drug-paired side during post- conditioning versus preconditioning tests (genotype $F_{(1, 37)} = 7.627$, $p < 0.01$; dose $F_{(2,37)} = 26.46$, $p < 0.0001$). (h) Distance traveled during the post-conditioning test show no difference between genotypes. Data represented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. $Rgs7^{+/+}$ group.

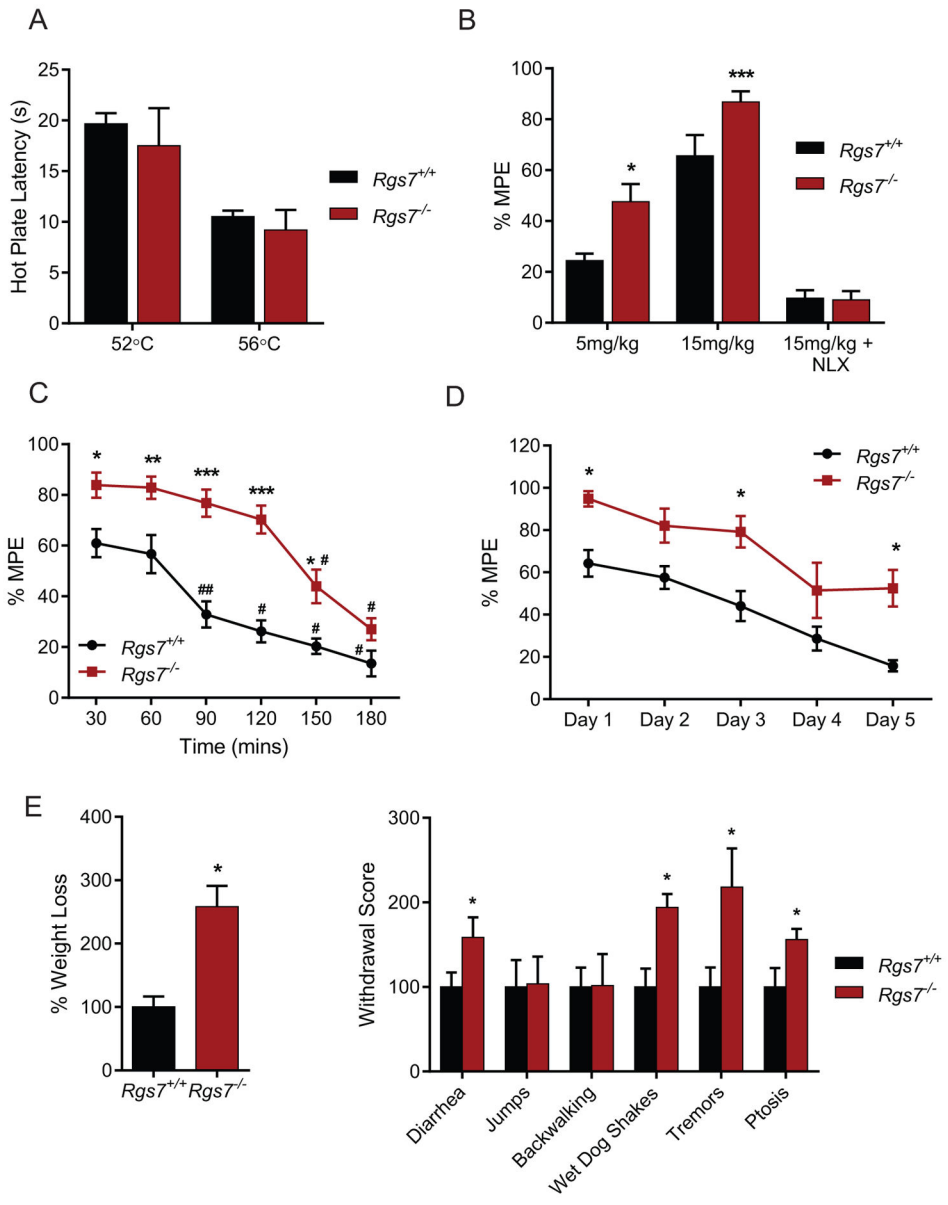

Figure 3. Global knockout of RGS7 affects analgesia, tolerance and withdrawal responses to morphine

(A) *Rgs7*$^{-/-}$ mice have comparable nociceptive threshold at 52°C and 56°C in the hot plate test as their wildtype littermates, *Rgs7*$^{+/+}$ mice (n = 9–15 per group). (B) *Rgs7*$^{-/-}$ mice have a heightened analgesia response to acute morphine treatment (5 mg/kg and 15 mg/kg) in the hot plate test. Naloxone (1 mg/kg, s.c.) blocks the morphine-induced analgesia response (n = 6–15 per group). Responses are expressed as a percentage of maximal possible effect (MPE = (latency-baseline)/(cutoff-baseline) (genotype $F_{(1, 49)} = 13.30$, $p < 0.001$; treatment $F_{(2, 49)} = 82.63$, $p < 0.0001$, interaction $F_{(2, 49)} = 3.466$, $p < 0.05$). (C) The duration of analgesia response to acute morphine treatment (15 mg/kg) measured every 30 min showed that *Rgs7*$^{-/-}$ mice has an extended effect over 180 min (genotype $F_{(1, 25)} = 50.58$, $p < 0.0001$; time $F_{(5, 125)} = 35.28$, $p < 0.0001$; interaction $F_{(5, 125)} = 3.182$, $p = 0.0097$). (D) *Rgs7*$^{-/-}$

mice show a delayed tolerance to morphine compared to *Rgs7*$^{+/+}$ mice (n = 8 per genotype). Mice were monitored by the hot plate test 30 min following morphine treatment (15 mg/kg) for 5 days (time $F_{(4, 56)} = 14.11$, $p < 0.0001$; genotype $F_{(1, 14)} = 41.08$ $p < 0.0001$). (E) Effects of morphine withdrawal on *Rgs7*$^{-/-}$ and *Rgs7*$^{+/+}$ mice. Mice are monitored over 30 min for weight loss, diarrhea, jumps, backwalking, wet dog shakes, tremor and ptosis following naloxone-precipitated morphine withdrawal (n = 8 per genotype). Data represented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. *Rgs7*$^{+/+}$ group; # $p < 0.0001$, ## $p < 0.001$ vs. respective 30 min time point.

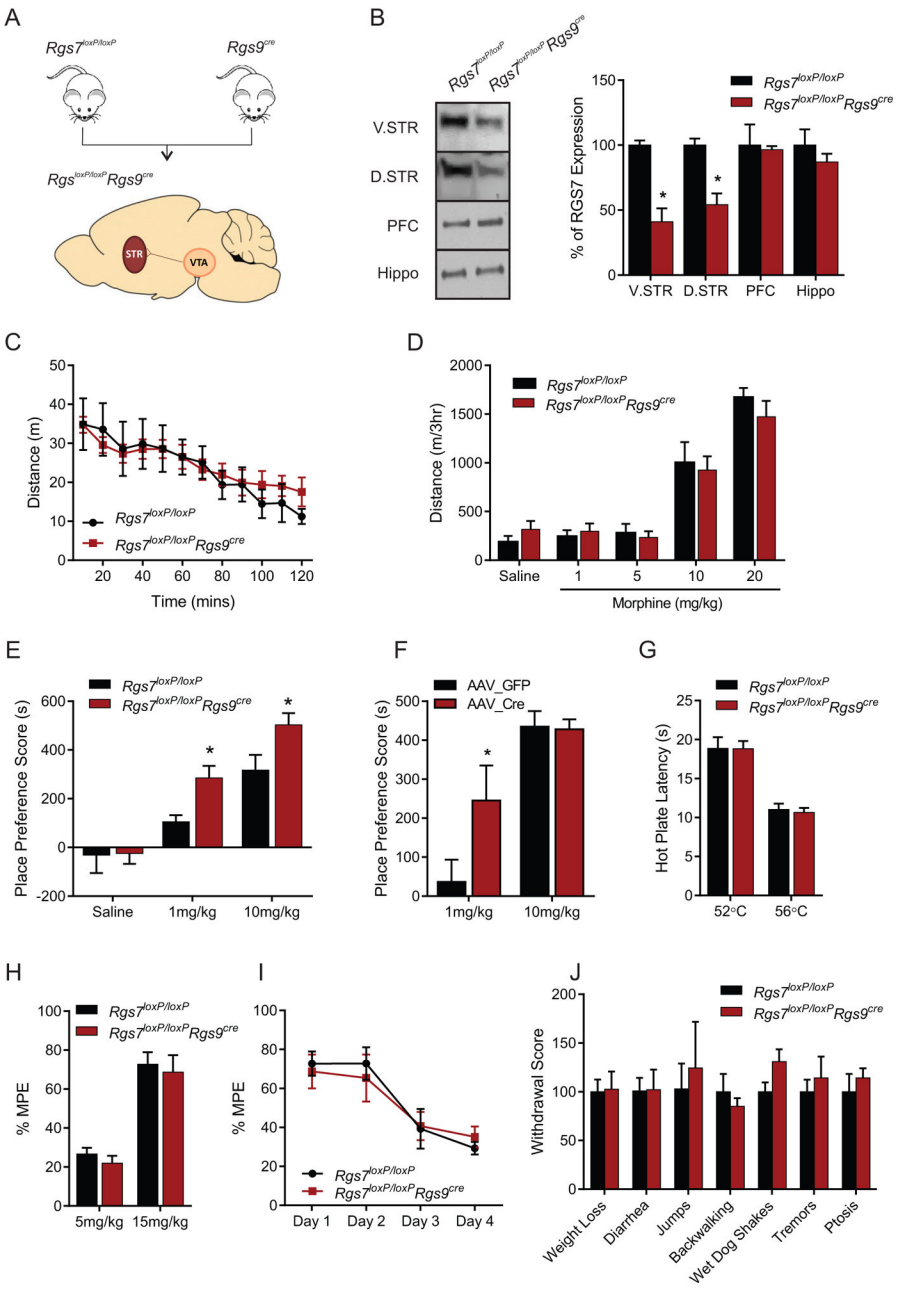

Figure 4. Elimination of RGS7 in striatal neurons affects morphine-induced conditioned place preference but not locomotion, analgesia and withdrawal

(A) Schematic for the generation of *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} mice. (B) Representative and summarized data of western blots showing that RGS7 levels in the ventral and dorsal STR were decreased in the *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} mice compared with the control mice. RGS7 levels in the prefrontal cortex (PFC) and hippocampus (Hippo) were not significantly different between the two genotypes (n = 4–6 per genotypes). (C) Locomotor activity of drug naïve mice (n = 5–7 per group) and (D) following administration of morphine (1, 5, 10 and 20 mg/kg) show no difference between *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} and *Rgs7*$^{loxP/loxP}$ mice (n = 8 per group). (E) Conditioned place preference show an enhanced place preference of

Rgs7$^{loxP/loxP}$*Rgs9*^{cre} mice in response to 1 mg/kg and 10 mg/kg morphine (n = 6–10 per group; genotype $F_{(1,43)} = 8.281$, $p < 0.01$, dose $F_{(2,43)} = 32.99$, $p < 0.0001$). (F) AAV_Cre injected into the NAc of *Rgs7*$^{loxP/loxP}$ mice show an enhanced place preference (1mg/kg morphine) compared to AAV_GFP mice (n = 4–5 per group; group $F_{(1,43)} = 26.00$, $p < 0.002$). (G) Hot plate latency show no difference in nociception at 52°C and 56°C between the genotypes (n = 6 per group). (H) Analgesia response to acute morphine treatment (5 mg/kg and 15mg/kg; n = 6–7 per group) and (I) repeated morphine treatment (15 mg/kg, n = 6 per group) in the hot plate test. (J) Effects of morphine withdrawal on *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} and *Rgs7*$^{loxP/loxP}$ mice (n = 8 per group). Data represented as mean ± SEM. * $p < 0.05$ vs. *Rgs7*$^{loxP/loxP}$.

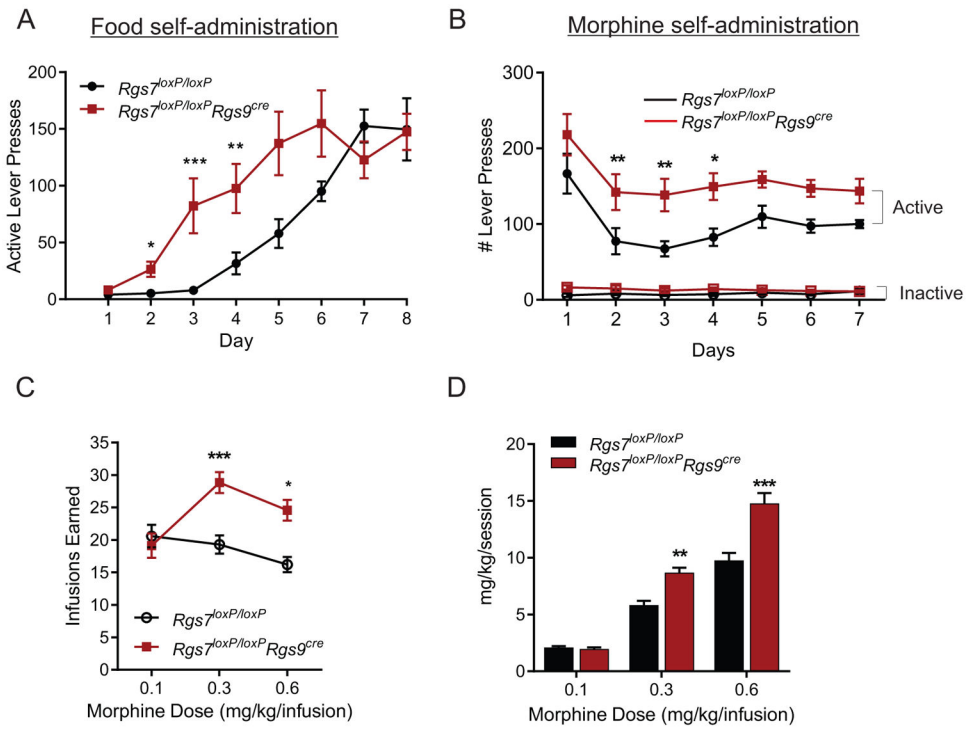

Figure 5. Deletion of RGS7 in the striatum increases morphine intake in self-administration task

(A) Number of active lever presses across an 8 day training in food self-administration paradigm for *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} and *Rgs7*$^{loxP/loxP}$ mice. Food training criteria was set under a fixed-ratio 5 (FR5) with a time out 20s (TO20s) schedule of reinforcement (genotype $F_{(1, 136)} = 22.45$, $p < 0.0001$; Day $F_{(7, 136)} = 36.32$, $p < 0.0001$; interaction $F_{(7, 136)} = 3.897$, $p < 0.001$). (B) Mice were then transitioned to 0.3 mg/kg/infusion of intravenous morphine (FR5, TO20s schedule of reinforcement) over 7 days and the number of active and inactive lever presses was recorded (genotype $F_{(1, 16)} = 20.23$, $p < 0.0004$; day $F_{(1, 136)} = 7.683$, $p < 0.0001$). (C) Number of infusions earned during morphine self-administration at doses of 0.1, 0.3 and 0.6 mg/kg/infusions (genotype $F_{(1, 16)} = 14.48$, $p < 0.005$; dose $F_{(2, 32)} = 4.410$, $p < 0.05$ and interaction $F_{(2, 32)} = 7.659$, $p < 0.01$). (D) Intake of morphine self-administration at morphine doses of 0.1, 0.3 and 0.6mg/kg/infusions calculated from the last three stable sessions (genotype $F_{(1, 16)} = 27.52$, $p < 0.0001$; intake $F_{(2, 32)} = 159.6$, $p < 0.0001$; interaction $F_{(2, 16)} = 10.19$, $p < 0.001$) . Data represented as mean ± SEM (n = 8–11/genotype). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. *Rgs7*$^{loxP/loxP}$.

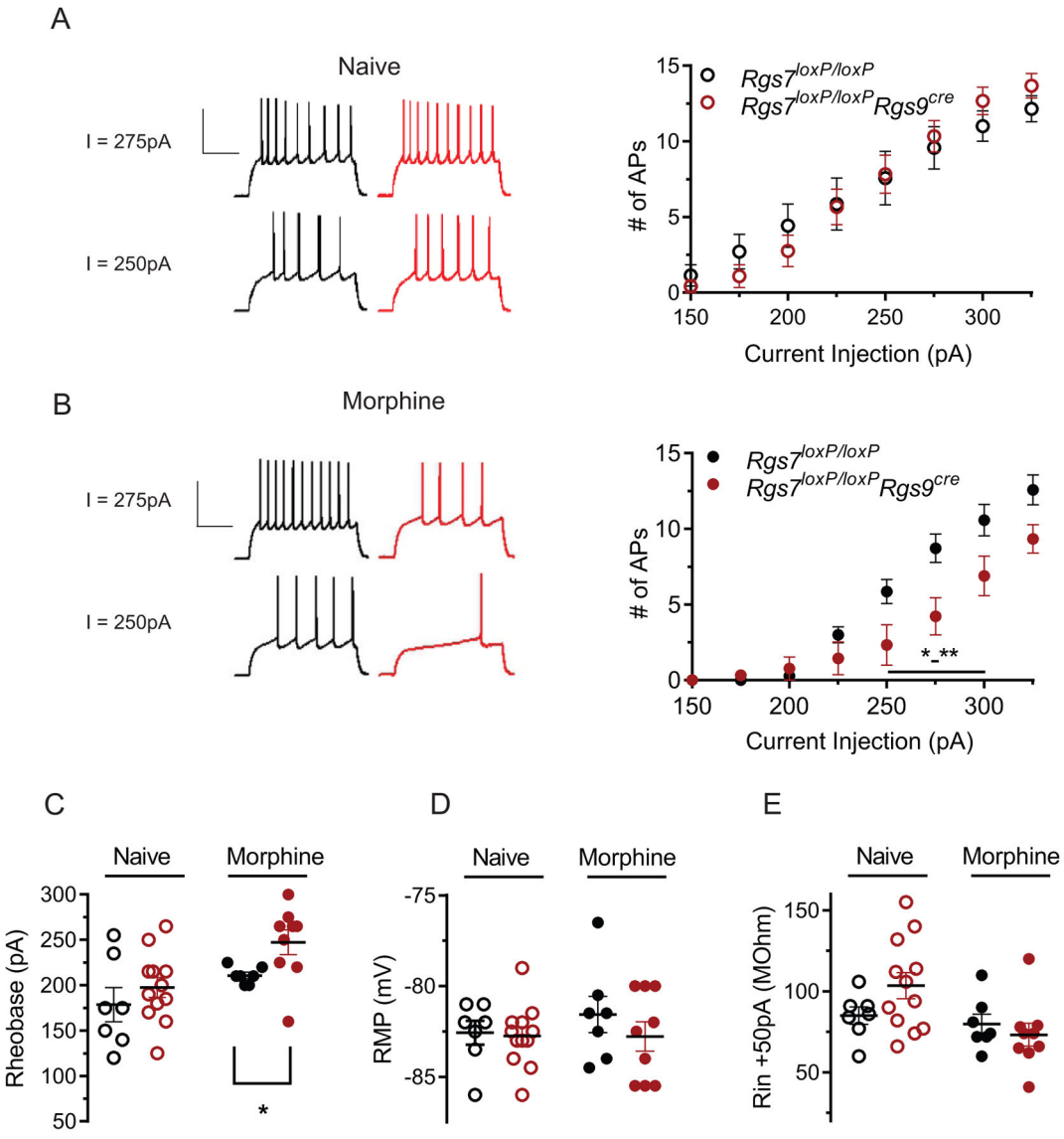

Figure 6. Morphine self-administration reduces excitability of MSN in NAc of mice lacking RGS7

(A) Representative traces of NAc MSN spiking activity in response to different levels of current injection in drug-naïve *Rgs7*$^{loxP/loxP}$ and *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} mice. Right panel shows relationship between the mean number of action potentials (APs) generated for a given level of current injection. (B) Representative traces of NAc MSN spiking activity in response to different levels of current injection in *Rgs7*$^{loxP/loxP}$ and *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} mice self-administering morphine. Right panel shows relationship between the mean number of APs generated for a given level of current injection (genotype $F_{(1, 14)} = 3.44$; injection $F_{(9, 126)} = 99.77$, $p < 0.0001$; interaction $F_{(9, 126)} = 5.80$, $p < 0.0001$). The mice used for these recordings represent a subset of mice studied in Fig. 6 analyzed 24 hours following the last self-administration session. (C) Comparison of the firing threshold (rheobase), (D) resting membrane potential (RMP) and (E) input resistance (R_{in}) for drug naïve and

morphine self-administrated *Rgs7*$^{loxP/loxP}$ and *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} mice. Scale bars: 250 ms, 50 mV.

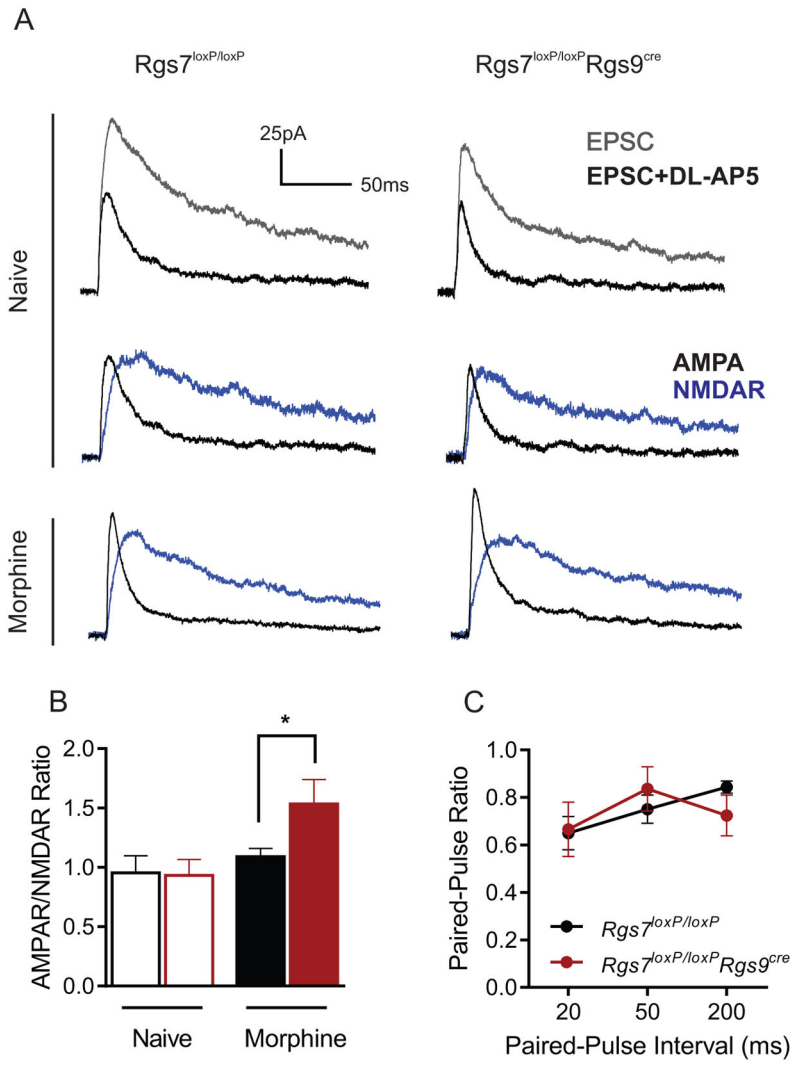

Figure 7. Striatal RGS7 gates morphine-induced changes in AMPA/NMDA receptor ratio in NAc MSN

(A) Representative traces of excitatory postsynaptic currents (EPSC) in MSN evoked by afferent stimulation in the NAc before (grey) and after (black) the addition of NMDA receptor blocker DL-AP5. Contribution of NMDA (blue) receptors to the response was determined by subtracting the AMPA receptor current from the total EPSC. Comparison of AMPA and NMDA receptor mediated currents was performed in drug-naïve mice as well as mice self-administering morphine for both *Rgs7*$^{loxP/loxP}$ and *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre}genotypes. (B) Calculation of AMPA to NMDA receptor ratios and their comparison across genotypes. T-test identifies significant increase in AMPAR/NMDAR ratio in *Rgs7*$^{loxP/loxP}$*Rgs9*^{cre} mice self-administering morphine (n=6–9 cells; *p < 0.05). (C) Lack of the genotype effect on paired pulse ratio in mice self-administering morphine.