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Nucleus Accumbens-Specific Interventions in RGS9-2 Activity Modulate Responses to Morphine

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Regulator of G protein signalling 9-2 (Rgs9-2) modulates the actions of a wide range of CNS-acting drugs by controlling signal transduction of several GPCRs in the striatum. RGS9-2 acts via a complex mechanism that involves interactions with Gα subunits, the Gβ5 protein, and the adaptor protein R7BP. Our recent work identified Rgs9-2 complexes in the striatum associated with acute or chronic exposures to mu opioid receptor (MOR) agonists. In this study we use several new genetic tools that allow manipulations of Rgs9-2 activity in particular brain regions of adult mice in order to better understand the mechanism via which this protein modulates opiate addiction and analgesia. We used adeno-associated viruses (AAVs) to express forms of Rgs9-2 in the dorsal and ventral striatum (nucleus accumbens, NAc) in order to examine the influence of this protein in morphine actions. Consistent with earlier behavioural findings from constitutive Rgs9 knockout mice, we show that Rgs9-2 actions in the NAc modulate morphine reward and dependence. Notably, Rgs9-2 in the NAc affects the analgesic actions of morphine as well as the development of analgesic tolerance. Using optogenetics we demonstrate that activation of ChannelrhodopsIn2 in Rgs9-2-expressing neurons, or in D1 dopamine receptor (Drd1)-enriched medium spiny neurons, accelerates the development of morphine tolerance, whereas activation of D2 dopamine receptor (Drd2)-enriched neurons does not significantly affect the development of tolerance. Together, these data provide new information on the signal transduction mechanisms underlying opiate actions in the NAc.
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INTRODUCTION

Regulator of G protein signalling 9-2 (Rgs9-2) is a 76-kD signal transduction modulator expressed in high levels in the striatum (Gold *et al*, 1997; Rahman *et al*, 2003; Traynor *et al*, 2009). Evidence from biochemical and *in vivo* studies suggests that Rgs9-2 complexes with Gα subunits, the Gβ5 protein, and other adaptor or scaffolding molecules dynamically modulate GPCR responsiveness and desensitization (Ballon *et al*, 2006; Anderson *et al*, 2007; Psifogeorgou *et al*, 2007; Terzi *et al*, 2009, Kimple *et al*, 2011; Masuho *et al*, 2011). Rgs9-2 complexes modulate the actions of a wide range of drugs, including opiate analgesics, psychostimulants, antipsychotic, and anti-parkinsonian medications (Rahman *et al*, 2003; Zachariou *et al*, 2003; Kovoor *et al*, 2005; Gold *et al*, 2007). In terms of opiate actions, Rgs9-2 complexes have been shown to affect several aspects of addiction, by negatively modulating reward and physical dependence; however, they also affect analgesia and the development of analgesic tolerance (Zachariou *et al*, 2003; Psifogeorgou *et al*, 2011).

Given the role of Rgs9-2 in addiction, it is essential to understand the brain region and cell type-specific actions of this protein in order to develop therapeutic approaches for the treatment of this disorder. It is particularly intriguing that Rgs9-2 complexes within the brain reward centre also have a part in morphine tolerance, as the NAc is not traditionally considered as an analgesia-modulating structure. Understanding the mechanism via which Rgs9-2 modulates the analgesic actions of opiates may provide important information for the prevention of morphine tolerance and the development of more efficient analgesics (Ling *et al*, 2011). It is therefore important to comprehend the brain region and cell type-specific actions of Rgs9-2, the composition and temporal regulation of Rgs9-2 complexes, as well as the context- and stimulus-dependent actions of

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such complexes. As a first step, in this study we apply genetic tools in order to understand the brain region-specific actions of Rgs9-2 in morphine-related behaviours. Specifically, we used stereotaxic surgery of adeno-associated viruses (AAVs) to overexpress Rgs9-2 in the NAc (or dorsal striatum) of adult C57BL\6 mice. Our findings suggest that increased Rgs9-2 activity in the NAc blocks morphine reward in the place preference paradigm and attenuates several signs of opiate withdrawal. Our findings also reveal that in the NAc, Rgs9-2 acts as a negative modulator of morphine analgesia and tolerance, confirming our hypothesis that cellular adaptations within the brain reward centre affect both addiction and analgesia. We also applied optogenetics in order to gain an understanding of the neuronal subpopulations in the striatum involved in morphine analgesic tolerance. We targeted neuronal populations expressing *Rgs9* in the NAc, or populations enriched in dopamine D1 receptors that project to the pallidal region and midbrain regions including substantia nigra and VTA (D1-type, direct pathway) and neuronal populations predominantly expressing dopamine D2 receptors (D2-type, indirect pathway), which project only to pallidal regions (Alexander *et al*, 1986; Graybiel, 1990; Smith *et al*, 2013). Our findings show that optogenetic activation of *Rgs9*-expressing neurons in the NAc leads to rapid development of analgesic tolerance to morphine in the hot plate test. Furthermore, activation of dopamine D1 receptor-expressing cells in the NAc increases RGS9-2 levels and accelerates the development of tolerance, whereas activation of the D2-type neurons has a minor effect on analgesic tolerance. Our data provide new information on the NAc-specific actions of Rgs9-2 and the signal transduction mechanisms involved in morphine reward, somatic withdrawal, and analgesia.

MATERIALS AND METHODS

Animals

Rgs9-Cre (Dang *et al*, 2006), Drd1-Cre (line FK150), Drd2-Cre (line ER44), and Drd1-tdTomato (line 5, Shuen *et al*, 2008)/Drd2-EGFP (line S118) lines were provided by Dr Nestler (Department of Neuroscience, Icahn School of Medicine at Mount Sinai) and Dr Heinz (Laboratory of Molecular Biology, Rockefeller University, gensat.org). Two- to three-month-old male C57BL\6 mice were used for viral-mediated gene transfer studies. Animals were housed in a 12-h dark/light cycle room according to the animal care and use committees of Icahn School of Medicine at Mount Sinai and the University of Crete.

Stereotaxic Surgery and Viral-mediated Gene Transfer

For all stereotaxic surgeries, mice were anaesthetized with Avertine (2,2,2-Tribromoethanol, Sigma). The AAV-EGFP, AAV-Rgs9-2-EGFP, and AAV-DEPlessRgs9-2 vectors were produced using a triple transfection helper-free method in HEK cells and purified, as described earlier (Hommel *et al*, 2003). The AAV-Cre-EGFP (EGFP-tagged Cre recombinase), DIO-AAV-ChR2-EYFP, and DIO-ChR2-EYFP vectors were provided by Dr Lobo (Lobo *et al*, 2010) and Dr Deisseroth (Stanford University). Stereotaxic coordinates for viral vector injections into the NAc were: AP $+1.6$ mm, LM $+1.5$ mm, and DV-4.4 mm at an angle of 10° from the midline and into the dorsal striatum AP $+1.6$ mm, LM $+1.5$ mm, and DV -3.4 mm at an angle of 10° from the midline. 0.5 μl of virus was bilaterally delivered over a 5-min period using a 33-gauge needle. The needle remained in place for 10 min post injection to ensure diffusion in nearby areas. Behavioural experiments were performed 2 weeks after AAV infection, a time point when transgene expression is maximal. For optogenetic studies, the microinjections were performed on the right hemisphere and the stereotaxic coordinates were modified in order to be able to guide the cannula and the optic fibers to the infection site (from bregma, angle 0°: AP -1.4 mm, Lat $+1.3$, DV -4.3). Two weeks after the viral infection, a 20-gauge cannula was implanted to the NAc (AP $+1.4$ mm, LM $+1.3$, DV -3.9) in order to guide the optic fibers. The cannula was secured to the scull using fixture adhesive (Leica) and then dental cement (Lang Dental). All cannulated animals were single-housed and tested in behavioural assays 2 days post cannulation.

In vivo Optogenetic stimulation

Optic fibers (Thor labs) of 200-μm core were attached to the cannula (inserted through the length of the cannula ~ 50 μm of the stripped 200-μm core exposed beyond the cannula) and connected through an FC/PC adaptor to a 473-nm blue laser diode (Crystal Lasers no. BCL-473-050-M) and a function generator (Agilent no. 33220A). The intensity of the optic stimulation was evaluated before every experiment using a light sensor (Thor Labs no. S130A). For the selected fibers, intensity ranged from 2 to 4 mW. The fibers were secured to the cannula immediately before the start of blue light stimulation. Light pulses were generated through the function generator. Optic fibers were removed from the cannula immediately after the end of the stimulation period.

Hot Plate Analgesia Assay

Analgesia was measured using a 52 °C hot plate apparatus (IITC Life Sciences, CA), as described (Psifogeorgou *et al*, 2011). Animals were habituated in the room for 1 h and then tested for baseline latencies. Morphine was injected s.c. (10, 20, or 30 mg/kg), and 30 min later mice were placed on the hot plate apparatus and latencies to lick the hind-paw or jump were monitored. For tolerance studies, this procedure was repeated for 4 or 5 consecutive days. All hot plate data are expressed as % maximal possible effect (MPE = [Latency − baseline]/[cutoff-baseline]). A cutoff time of 40 s has been used in all hot plate experiments to avoid tissue damage and inflammation.

Optogenetic Studies for Hot Plate Assays

For the optogenetic studies, adult male heterozygote Rgs9-Cre, Drd1-Cre, and Drd2-Cre mice infected with DIO-AAV-ChR2 or control DIO-AAV-EYFP received blue-light stimulation (10-Hz light pulses (100 ms square width) for 30 s for 3 min, every 3 min for a 20 min period, Lobo *et al*, 2010; Chandra *et al*, 2013) after baseline hot plate

monitoring and s.c. morphine injection (20 mg/kg i.p.). Hot plate latencies were measured again 30 min post-morphine injection. This procedure was repeated for 4 consecutive days. A separate group of Drd1-Cre and Drd2-Cre mice expressing DIO-AAV-ChR2 or control DIO-AAV-EYFP in the NAc were used for western blot analysis studies. The optogenetic protocol described above was applied in morphine- or saline-treated mice, and NAc was dissected on Day 3, 1 h after the last hot plate trial.

Conditioned Place Preference test

An unbiased place-conditioning procedure was performed as described in earlier studies (Han *et al*, 2010). Briefly, after monitoring baseline preference on day 1 for 20 min, animals were conditioned to the saline-paired side for 30 min in the morning and to the drug-paired side for 30 min in the afternoon. After three conditioning sessions animals were tested for 20 min, and preference was determined as time spent in the drug-paired compartment after conditioning minus time spend in the drug-paired compartment at baseline. Animals showing strong bias (over 250 s difference in the time spent between the two sides) for one side at baseline were excluded from the study. On the basis of these criteria, six animals from the saline or morphine groups were excluded from our CPP experiments.

Fear Conditioning

Mice were habituated for 2 days in testing room, and on day 3 they were trained with a single foot shock (the foot shock is applied for 2 s and 2 min after the mouse is placed in the chamber). The total time spent in the fear-conditioning chamber is 3 min. On day 4 the exact same procedure takes place but without a foot shock, and behavior is monitored in order to determine freezing time. Freezing time is expressed as the percentage of total time spent in the chamber.

Opiate withdrawal paradigm

For opiate withdrawal assays, mice were injected with increasing morphine doses every 12 h (Terzi *et al*, 2012) for 4 consecutive days (day 1: 20 mg/kg, day 2: 40 mg/kg, day 3: 60 mg/kg, day 4: 80 mg/kg) and on the fifth day they were injected with 100 mg/kg morphine in the morning and withdrawal was precipitated 3 h after the last injection using naloxone hydrochloride (1 mg/kg, s.c., Sigma, MO). Withdrawal signs were monitored for 25 min after naloxone administration. Data were expressed as number of jumps, wet dog shakes, and diarhhea observed in the 25-min period, and % change in weight from before naloxone administration to 30 min post withdrawal. For tremor and ptosis we monitored the presence of a sign at the beginning of each 5-min interval during the 25-min monitoring period.

Immunofluorescence studies

Mice were anesthetized with chloral hydrate and then perfused for 3 min with phosphate buffered saline (PBS) followed by 12 min with 4% paraformaldehyde (PFA). Brains were stored in PFA for 6 h and then transferred to 1× PBS-30% glycerol. For immunofluorescence studies, 4-μm brain sections were obtained with a microtome (Leica). To identify EGFP expression, the slices were dehydrated and mounted, and the viral expression pattern was visualized using a fluorescence microscope (ZEISS AXIO) with ×5 and ×10 objectives (Lobo *et al*, 2010).

Western blot analysis

For western blot analysis, NAc punches were dissected with a 12-gauge syringe needle from 1-mm-thick coronal sections of mouse brain, as described before (Charlton *et al*, 2008). Samples were run on a 10% polyacrylamide gel, and then transferred to a nitrocellulose membrane (Bio-Rad, 4.45 μm) for 1 h. Membranes were incubated in blocking buffer (3% filtered non-fat dry milk, in PBS) and incubated overnight at 4 °C in PBS with primary antibody. The following antibodies were used: rabbit anti-RGS9-2 antibody (Psifogeorgou *et al*, 2011), a rabbit anti-Gβ5 (C-terminus) antibody (W. Simonds, NIDDKD), and a mouse anti-GADPH antibody (Abcam). Gβ5 or GADPH were used as loading controls. The next day membranes were incubated with goat anti-rabbit peroxidase-labelled secondary antibody (Invitrogen, 1 : 20 000) in blocking buffer. The bands were visualized using Super Signal West Pico Extended Duration Substrate (Pierce). Bands were quantified using the Image J Software.

Fluorescence-activated cell sorting (FACS) and qPCR

The 12-gauge NAc punches from *drd1a*-tdTomato/*drd2*-GFP transgenic mice were enzymatically dissociated using papain (Worthington) and a pure population of drd1-tdTomato + or drd2-GFP + MSNs was obtained via FACS using a previously published protocol (Lobo *et al*, 2010). RNA was extracted using the PicoPure RNA Isolation Kit (Life Technologies) including a DNase I digestion according to the manufacturer's protocol. RNA quality and relative concentration were confirmed on the Picochip using Agilent 2100 Bioanalyzer. cDNA was generated using an iScript Kit (Bio-Rad) according to the manufacturer's protocol. Quantitative PCR was carried out on a 7900HT using SYBR green. Each reaction was run in triplicate and analyzed following the standard $\Delta\Delta C_t$ method using 18S rRNA as a normalization control (Sun *et al*, 2012).

Statistical Analysis

For place preference, opiate withdrawal, and morphine tolerance studies, we used two-way ANOVA followed by Bonferroni *post hoc* test. For acute analgesia assays, we used *t*-test. For western blot analysis studies, comparisons between two experimental groups were performed using *t*-test and comparison for larger groups were performed using one-way ANOVA followed by Dunnett *post hoc* test. For qPCR analysis we used *t*-test. Main and interaction effects were considered significant at $P<0.05$; all data are expressed as mean ± SEM.

RESULTS

Our previous work demonstrated that genetic ablation of *Rgs9* leads to a tenfold increase in morphine reward sensitivity in the place preference paradigm (Zachariou *et al*, 2003). Here, we applied viral-mediated gene transfer methodology to infect the NAc of adult C57BL\6 mice with AAV vectors expressing Rgs9-2 and assessed the impact of this genetic intervention in morphine place preference. Consistent with the hypothesis that Rgs9-2 acts as a negative modulator of morphine reward, increased Rgs9-2 expression in the NAc following infection with AAV-Rgs9-2 vectors prevents morphine place preference. As shown in Figure 1a, while control AAV-GFP-infected C57BL\6 mice develop place preference to morphine at a dose of 5 mg/kg, mice overexpressing Rgs9-2 in the NAc show no place conditioning to 5 or 10 mg/kg of s.c. morphine. Saline responses in the CPP tests were not different between genotypes (Figure 1b). Figure 1c shows representative Rgs9-2 western blots from NAc tissue of mice infected with AAV-GFP and AAA-Rgs9-2, 3 weeks post-stereotaxic surgery. Infection with AAV-Rgs9-2 leads to a $74 \pm 20\%$ increase in Rgs9-2 expression in the NAc. Figure 1c shows a representative fluorescence picture from a NAc slice 3 weeks post-AAV-GFP infection. Notably, AAV-RGS9-2 infection does not have a significant effect on endogenous Rgs9-2 levels outside the infection site (Rgs9-2 OD in punches lateral to the infection area were: AAV-GFP group $= 1.18 \pm 0.46$ and AAV-Rgs9-2 group $= 2.1 \pm 0.69$). Overexpression of Rgs9-2 in the NAc did not produce any learning deficits in the animals, as assessed by the contextual fear-conditioning paradigm (Supplementary Figure 1a).

Previous studies from our group showed that chronic exposure to high doses of morphine (by use of s.c. morphine pellets) leads to a 50% decrease in Rgs9-2 levels in the NAc (Zachariou *et al*, 2003). We used bacterial artificial chromosome (BAC) transgenic mouse lines expressing tdTomato or EGFP under the control of dopamine receptor D1 (Drd1) or dopamine receptor D2 (Drd2) promoter and collected dopamine D1 or dopamine D2 expressing NAc medium spiny neurons using FACS (Lobo *et al*, 2006, 2010). We then performed qPCR to evaluate *Rgs9* expression levels in each of these neuronal populations in morphine-dependent mice, using 18S rRNA as a control. Our data indicate that *Rgs9* is selectively downregulated in D2-type neurons 5 days after exposure to high morphine doses (25 mg s.c. pellet on day 1 and a second 25-mg pellet on day 3), which lead to severe dependence (Figure 2a). Control experiments for the FACS analysis studies are shown in Supplementary Figure 2.

Our earlier work on constitutive knockout mice demonstrated that Rgs9-2 affects morphine physical dependence (Zachariou *et al*, 2003). On the basis of the above findings we hypothesized that increased Rgs9-2 activity in the NAc opposes the development of physical dependence and ameliorates somatic signs of morphine withdrawal. Consistent with this hypothesis, overexpression of Rgs9-2 in the NAc via stereotaxic injection of AAV-Rgs9-2 vectors reduces several symptoms of morphine withdrawal, including wet dog shakes, diarrhea, and weight loss (Figure 2b). The number of jumps was not different between genotypes

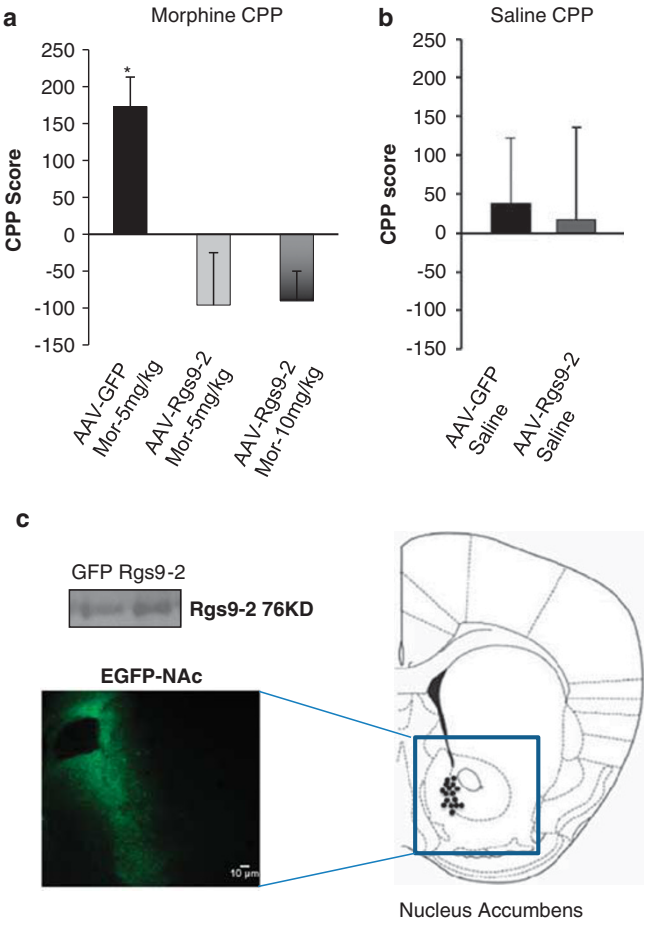

Figure 1 Increased regulator of G protein signalling 9-2 (Rgs9-2) activity in the nucleus accumbens (NAc) blocks morphine reward. Mice infected with adeno-associated viruses (AAV)-Rgs9-2 or control AAV-GFP constructs in the NAc via stereotaxic surgery were tested in the CPP paradigm. As shown in Figure 1a, overexpression of Rgs9-2 in the NAc prevents place morphine-conditioned place preference (Mor, 5 or 10 mg/kg s.c. $n = 6$–7 per group, $^*P < 0.01$ two-way ANOVA followed by Bonferroni *post hoc* test). Saline injections did not produce place preference in wild-type or mutant mice (Figure 1b, $n = 8$ per group). Figure 1c shows western blot analysis of Rgs9-2 levels in the NAc of AAV-GFP and AAV-Rgs9-2-infected mice and the distribution of EGFP in the NAc (× 10 ZEISS AXIO Fluorescent Microscope), 3 weeks post-viral infection. Placements were circumscribed within the dorsomedial part of the NAc, including parts of both shell and core at A/P coordinates between + 1.5 and + 1.7 from bregma.

(jumps for AAV-GFP $= 42 \pm 6.7$ and for AAV-Rgs9-2 $= 43 \pm 7.4$). Naloxone had no effect on saline-treated AAF-GFP or AAV-Rgs9-2 NAc overexpressors (withdrawal signs for AAV-GFP group: jumps $= 0 \pm 0$, wet dog shakes $= 1 \pm 0.36$, tremor $= 0 \pm 0$ ptosis $= 0 \pm 0$, diarrhea $= 2.4 \pm 0.66$, and weight loss $= 0.48 \pm 0.13$ and for AAV-Rgs9-2 group: jumps $= 0 \pm 0$, wet dog shakes $= 1.25 \pm 0.19$, tremor $= 0 \pm 0$, ptosis $= 0 \pm 0$, diarrhea $= 1.5 \pm 0.22$, and weight loss $= 0.58 \pm 0.2$). To show that these actions are not a general result of Rgs9-2 vector infection in the brain, we infected the dorsomedial striatum of C57BL\6 mice with AAV-Rgs9-2 or control AAV-GFP vectors (See Supplementary Figure 3). Rgs9-2 overexpression in the dorsomedial striatum had a

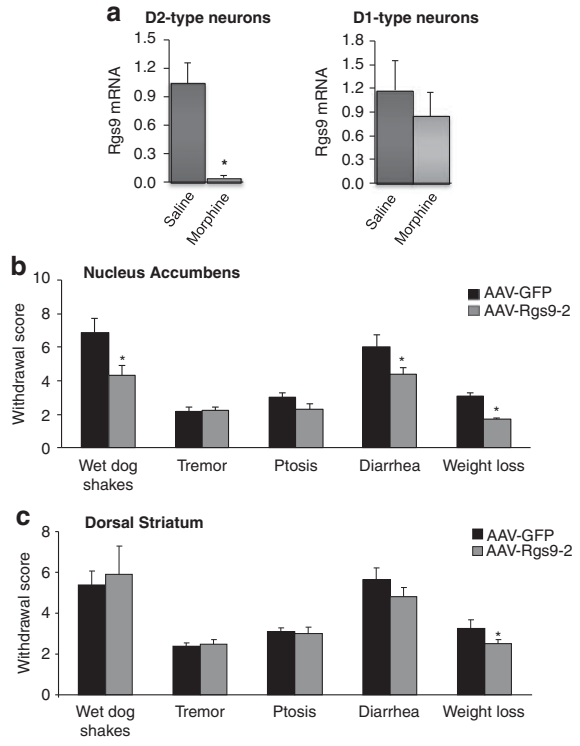

Figure 2 A role of regulator of G protein signalling 9-2 (Rgs9-2) in morphine withdrawal. qPCR analysis from Fluorescence-activated cell sorting (FACS)-sorted GFP-positive D1 or D2 type striatal neurons reveals that chronic morphine (25 mg pellets, on days 1 and 3 of a 5 day paradigm) leads to a robust downregulation of *Rgs9* expression in D2 type but not in D1 type neurons (Figures 2a, $n=3-4$ per group, $^{*}P<0.05$, *t*-test). Mice were infected with the adeno-associated viruses (AAV)-Rgs9-2 or AAV-GFP constructs in the nucleus accumbens (NAc) and used in a morphine withdrawal paradigm 2 weeks after surgery. In Figure 2b, Animals were injected with increasing morphine doses for 4 days, and on day 5 morphine withdrawal was precipitated by naloxone injection (1 mg/kg s.c.). Increased Rgs9-2 activity in the NAc significantly reduces the number of wet dog shakes, diarhhea occurrence, and % weight loss. Interestingly, overexpression of Rgs9-2 in the dorsal striatum had a small but significant effect on weight loss (Figure 2c, $n=9-12$ per group). Data are expressed as mean ± SEM. $^{*}P<0.05$, two-way ANOVA followed by Bonferroni test.

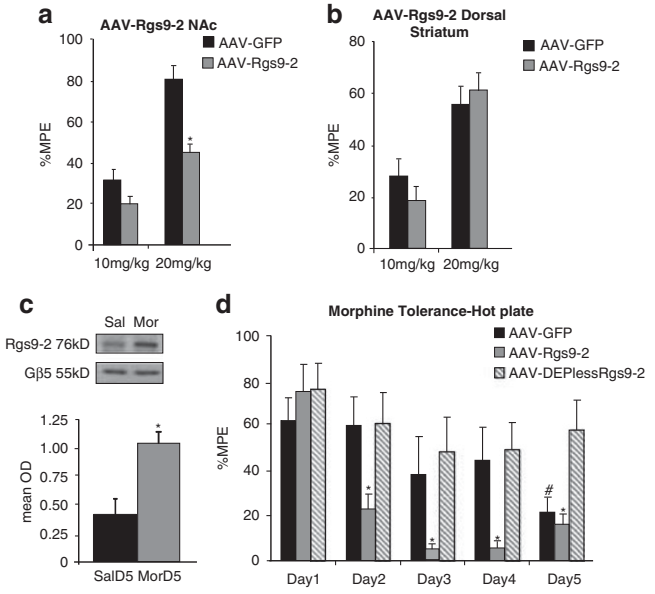

Figure 3 Regulator of G protein signalling 9-2 (Rgs9-2) actions in the nucleus accumbens (NAc) modulate morphine analgesia and tolerance. Mice infected with adeno-associated viruses (AAV)-Rgs9-2 or AAV-GFP constructs in the NAc were evaluated in the hot plate paradigm in order to determine the role of Rgs9-2 in the NAc in analgesic responses to morphine. As shown in Figure 3a, overexpression of Rgs9-2 in the NAc reduces the analgesic actions of morphine in the 52 °C hot plate test ($n=10$ per group, $^{*}P<0.05$, *t*-test). Overexpression of Rgs9-2 in the dorsomedial striatum does not affect hot plate responses to morphine (Figure 3b, $n=10$ per group). In Figure 3c, western blot analysis studies reveal that Rgs9-2 expression in the NAc increases by the fifth day of morphine (Mor) administration in a hot plate tolerance paradigm ($P<0.01$ *t*-test, $n=5$ per group, Sal = Saline). A separate group of animals received a high morphine dose (30 mg/kg i.p.) and were evaluated in the hot plate test for 5 consecutive days. As shown in Figure 3d, overexpression of Rgs9-2 in the NAc accelerates the development of morphine tolerance, whereas expression of the dominant-negative DEPlessRgs9-2 in the NAc delays the development of tolerance ($n=7-8$ per group, $^{*}P<0.05$ for over-expressors *vs* AAV-GFP controls, $^{\#}P<0.05$ for AAV-GFP day 5 *vs* day 1 and for AAV-GFP day 5 *vs* AAV-DEPlessRgs9-2 day 5). All data are expressed as means ± SEM, two-way ANOVA followed by Bonferroni *post hoc* test.

small but significant effect on weight loss, suggesting that GPCRs in this brain region have a small influence on morphine withdrawal (Figure 2c).

Morphine possesses strong analgesic actions, which are mediated via activation of MOR in various brain regions (Contet *et al* 2004). Recently, we have demonstrated that interventions in signal transduction events at the level of the NAc may affect not only the rewarding but also the analgesic actions of morphine (Zachariou *et al*, 2006; Psifogeorgou *et al*, 2011). The initial characterization of *Rgs*9KO mice (Zachariou *et al*, 2003) revealed that deletion of the *Rgs*9 gene delays the development of analgesic tolerance to morphine. This phenotype was attributed to Rgs9-2 actions in the striatum; however, the presence of Rgs9-2 in low amounts in other CNS regions such as the dorsal horn of the spinal cord or the hippocampus cannot exclude the possibility of Rgs9-2 actions through extra-striatal networks. To demonstrate that increased Rgs9-2 activity in the NAc of adult animals modulates the analgesic actions of morphine, we expressed AAV-Rgs9-2 in the NAc of C57BL\6 mice and evaluated animals in the hot plate paradigm. We administered a low morphine dose, known to have a minor analgesic effect in the hot plate assay (10 mg/kg) and a higher dose (20 mg/kg) that produces maximal analgesia in C57BL\6 mice. As shown in Figure 3a, increased Rgs9-2 activity in the NAc reduces the analgesic actions of morphine in the hot plate assay. On the contrary, infection of the dorsal striatum with AAV-Rgs9-2 did not affect responses to morphine in the hot plate test (Figure 3b). We next used western blot analysis to monitor Rgs9-2 expression in the NAc of C57BL\6 mice, 2 h after the last session of the morphine tolerance hot plate assay. As shown in Figure 3c, on the 5th day of morphine administration, when mice show a poor analgesic response due to the development of tolerance, Rgs9-2 levels are increased ~50% (OD for the Saline group = 0.39 ± 0.14 and for the

Morphine group = 1.01 ± 0.11). To examine our hypothesis that Rgs9-2 complexes in the NAc modulate morphine tolerance, we overexpressed Rgs9-2 in the NAc of adult C57BL\6 mice via stereotaxic infection with the AAV-Rgs9-2 vector. To locally antagonize Rgs9-2 activity in the NAc, we used an AAV vector expressing the mutant form DEPlessRgs9-2 that lacks the N-terminal DEP domain. The DEP domain is necessary for attachment to the cell membrane via interactions with the adaptor molecule R7BP (Ballon *et al*, 2006). DEPlessRgs9-2 has a diffuse cytoplasmic distribution and acts as dominant-negative by antagonizing endogenous Rgs9-2 interactions (Psifogeorgou *et al*, 2007). Expression levels of DEPlessRgs9-2 were measured using western blot analysis (Supplementary Figure 3). Animals were infected in the NAc with the AAV-Rgs9-2, AAVDEPlessRgs9-2, or control AAV-GFP vectors and the 52 °C hot plate assay was performed 2 weeks later. As shown in Figure 3d, Rgs9-2 actions in the NAc have a potent role in the development of morphine tolerance: mice overexpressing Rgs9-2 in this brain region show tolerance by the second day of treatment, whereas their AAV-GFP-infected controls develop tolerance by the fifth treatment day. No differences in hot plate responses were observed in control saline-treated groups (Supplementary Figure 1). As indicated in the same figure, overexpression of the dominant-negative AAV-DEPlessRgs9-2 construct in the NAc delays the development of morphine tolerance (Figure 3d).

To further understand the role of NAc in morphine tolerance, we applied an optogenetic approach that permits activation of specific NAc subpopulations. We first used a transgenic line that expresses Cre recombinase under the control of the *Rgs*9 promoter (Dang *et al*, 2006). As Rgs9-2 is expressed in all types of striatal neurons (Gold *et al*, 1997) in this mouse line Cre recombinase is expressed in all neuronal subpopulations in the striatum. These animals were infected in the NAc with the Cre-inducible DIO-AAV-ChR2-EYFP construct, to express channelrhodopsin2 (ChR2), a blue light-sensitive cation channel opsin (Yizhar *et al*, 2011). To test morphine analgesia, mice received blue light illumination (10-Hz light pulses for 30 s for 3 min, every 3 min for a 20-min period, see Materials and Methods) after baseline hot plate monitoring and s.c. morphine injection. Animals were tested on the hot plate 30 min post-morphine injection. Control animals were infected with a DIO-AAV-EYFP construct and also received blue light illumination to the NAc. As shown in Figure 4a, activation of the ChR2 gene in *Rgs9*-expressing cells in the NAc leads to earlier development of tolerance. To investigate whether the tolerance phenotype is related to changes in the activity of specific NAc neuronal populations we used two BAC transgenic Cre lines to target the main subpopulations of medium spiny neurons. To target neurons of the direct dopamine pathway (Drd1, dopamine receptor 1-enriched neurons or D1-type neurons), we used the Drd1-Cre BAC transgenic line, and to target the neurons of the indirect pathway (Drd2, dopamine receptor D2-expressing neurons or D2-type) we used the Drd2-Cre BAC line. Adult male mice from each line were injected with the DIO-AAV-ChR2-EYFP or control DIO- AAV-EYFP vector into the NAc. Mice received blue-light illumination to the NAc of Drd1-Cre or Drd2-Cre mice infected with either DIO-AAV-ChR2-EYFP or with control DIO-AAV-EYFP construct immediately after baseline measurement and morphine injection. Persistent activation of D2-type neurons has no significant effect on morphine responses in the hot plate tolerance paradigm (Figure 4b) and does not affect Rgs9-2 levels (Supplementary Figure 4a). A more prolonged stimulation protocol (3 min every 3 min for 20 min) also failed to affect morphine analgesia or tolerance. On the other hand, increased neuronal activity in D1-type neurons leads to faster development of morphine tolerance (Figure 1c). Notably, this increased neuronal activation leads to an adaptive increase in Rgs9-2 levels (Figure 4d). As shown in Figure 3c, a similar increase is observed in C57BL\6 mice on the fourth day of the hot plate assay, when animals become tolerant to morphine. This is the first evidence of a role of direct pathway neurons in the development of morphine tolerance. Notably, vehicle injection does not affect hot plate responses to morphine in the Drd1 or Drd2 Cre line. (Latencies for the Drd1 Cre line in the hot plate test: for EGFP group, Day 1 = 9.5 ± 0.96, Day 2 = 11.7 ± 1, and Day 3 = 10.9 ± 2.4, for the ChR2 group, Day 1 = 11.7 ± 1.5, Day 2 = 9.9 ± 1.2, and Day 3 = 8.4 ± .13. Latencies for Drd2 Cre line, EYFP group Day 1 = 9.4 ± 2.6, Day 2 = 11.9 ± 2.6, Day 3 = 14.8 ± 3.1; for ChR2 group Day 1 = 11.3 ± 0.9, Day 2 = 12.4 ± 2, and Day 3 = 13.2 ± 0.2).

DISCUSSION

Using AAV vectors for highly localized interventions in Rgs9-2 activity in the NAc of adult mice, we demonstrate a potent role of Rgs9-2 in morphine reward, somatic morphine withdrawal behaviours, as well as in morphine analgesia and analgesic tolerance. These data further support the findings from constitutive *Rgs9* knockout mice showing that Rgs9-2 is a negative modulator of morphine actions (Zachariou *et al*, 2003; Psifogeorgou *et al*, 2011; Xie *et al*, 2012).

Over the last few years, a number of studies have elucidated the mechanisms underlying the cellular actions of opiates and provided important new information about signal transduction events associated with addiction or the development of analgesic tolerance. Most of these studies were based on genetic mouse models and revealed the consequences of global deletion of genes such as beta arrestin 2, spinophilin, or PLCβ3 in the acute and chronic actions of opiates (Xie *et al*, 1999; Charlton *et al*, 2008; Quillinan *et al*, 2011; Raehal and Bohn 2011). Given that signal transduction complexes modulate neuronal responses in a cell type and brain region-specific manner, there is a need for models that permit local manipulations in gene expression. Gene transfer approaches, conditional knockout mice, and targeted pharmacological interventions can be used to selectively manipulate the function/activity of molecules implicated in mu opioid receptor (MOR) signalling and desensitization. Such cell type or brain region-targeting approaches have provided novel information on the mechanisms underlying CNS disorders, including depression and addiction (Lobo *et al*, 2010; Koo *et al*, 2012; Lim *et al*, 2012; Chaudhury *et al*, 2013; Tye *et al*, 2013).

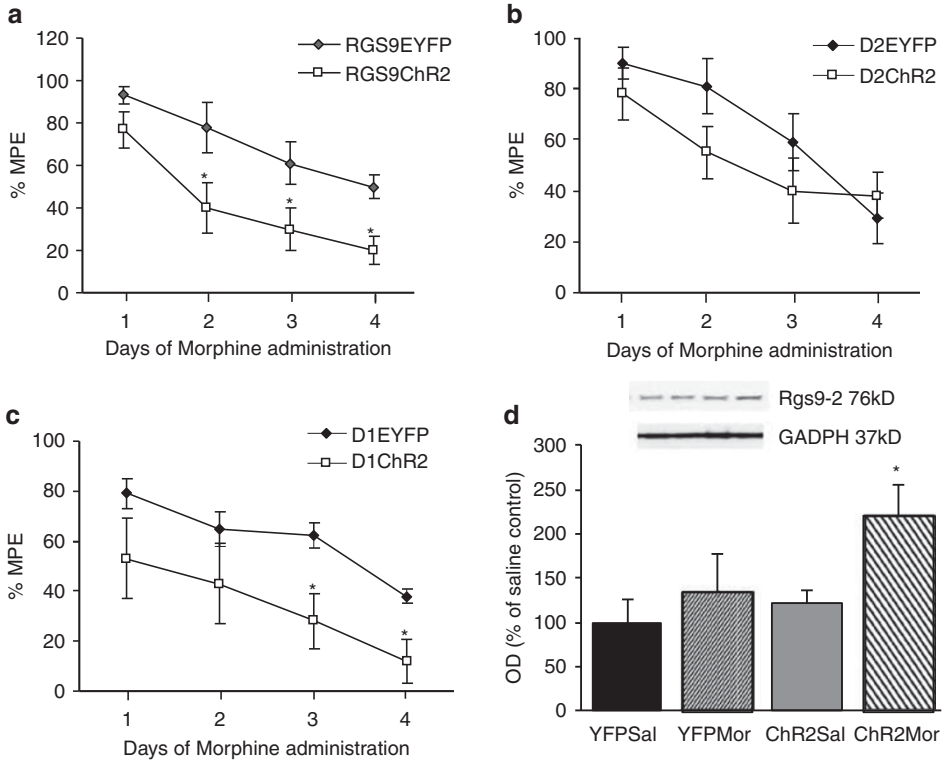

Figure 4 Optogenetic activation of neuronal subpopulations in the nucleus accumbens (NAc) modulates morphine tolerance. Optogenetic activation of regulator of G protein signalling 9-2 (*Rgs9*)-expressing neurons in the NAc accelerated the development of morphine tolerance in the hot plate paradigm (Figure 4a, $n=8$ per group, $P<0.05$, two-way ANOVA followed by Bonferroni *post hoc* test). In Figure 4b, Drd2-Cre mice were infected in the NAc with inverted double floxed DIO-AAV (adeno-associated viruses)-ChR2-EYFP or control DIO-AAV-EYFP vectors. Optogenetic activation of D2-type neurons in the NAc does not have a significant effect on the development of morphine tolerance ($n=5-6$ per group). Drd1-Cre mice were infected in the NAc with inverted double floxed DIO-AAV-ChR2-EYFP or control DIO-AAV-EYFP vectors. As shown in Figure 4c, optogenetic activation of D1-type neurons in the NAc accelerates the development of morphine tolerance $n=5-7$ per group, $^{*}P<0.05$ two-sway ANOVA followed by Bonferroni *post hoc* test. A separate group of DIO-AAV-ChR2-EYFP or control DIO-AAV-EYFP injected mice were optogenetically activated until day 3 of morphine (or saline) administration in hot plate tolerance assay and the NAc was dissected 1 h after the last hot plate assay (Sal = Saline, Mor = morphine). Western blot analysis reveals that persistent activation of the NAc in morphine-treated DIO-AAV-ChR2-EYFP-expressing mice increases Rgs9-2 levels (Figures 4d, $n=3-5$ per group $^{*}P<0.05$, one-way ANOVA, followed by Dunnett test).

Our viral expression protocols permitted us to increase Rgs9-2 activity in the NAc of adult mice in order to prevent the rewarding effect of morphine. Although morphine has strong rewarding actions, overexpression of Rgs9-2 in the NAc blocks morphine CPP. Interestingly, Rgs9-2 overexpressors fail to acquire conditioned place preference to morphine even when the drug is administered at high doses. Indeed, at both 5 and 10 mg/kg groups there was a trend for conditioned place aversion; however this trend was not significant. These findings are in accordance with earlier observations of a tenfold increase in morphine reward sensitivity in *Rgs9* knockout mice. Rgs9-2 overexpression in the NAc also attenuates some of the most prominent morphine withdrawal symptoms suggesting a role of Rgs9-2 in modulation of aberrant dopamine function under withdrawal conditions. On the other hand, increased Rgs9-2 activity in the dorsomedial striatum had a minor effect on withdrawal behaviours. Whereas these findings suggest that this striatal subregion has no major role in morphine withdrawal, it is possible that a broader overexpression of Rgs9-2 or overexpression in the dorsolateral striatum may reveal a role in morphine dependence. By monitoring *Rgs9* expression in the two main medium spiny neuronal populations in the striatum, we found that conditions leading to severe morphine dependence dramatically decrease Rgs9 gene expression in D2-type neurons. This is the first evidence of brain region and cell type-selective adaptations of *Rgs9* expression in a model of opiate addiction. Earlier western blot analysis studies from the NAc tissue have demonstrated that Rgs9-2 protein levels are downregulated in mice receiving the same morphine pellet treatment (Zachariou *et al*, 2003). This downregulation is perceived as an adaptive response in advanced addiction stages where severe alterations in synaptic function and neuronal morphology are observed (Bailey and Connor, 2005). As Rgs9-2 modulates the function of various GPCRs in the NAc, this adaptive response to chronic morphine may affect several receptors in the indirect pathway. We hypothesize that this downregulation of Rgs9-2 in D2-type neurons enhances Gi/o signal transduction. This robust cell type-specific downregulation is the first step towards understanding Rgs9-2 actions in the NAc under conditions of morphine dependence. Whereas these data point to a D2-type cell-specific regulation of Rgs9-2, the fact that Rgs9-2 levels do not change in D1–type neurons under these conditions does not exclude the possibility that Rgs9-2

protein interactions in the D1 population modulate morphine dependence. Current efforts are targeted towards the development of genetic models for manipulations of Rgs9-2 activity in specific subtypes of medium spiny neurons, in order to further comprehend the cell type-specific mechanism via which Rgs9-2 complexes modulate the acute and chronic actions of psychoactive substances.

Our findings also reveal a role of NAc Rgs9-2 in morphine analgesia and tolerance. These findings are complemented by optogenetic studies demonstrating a role for D1-type receptors in tolerance to morphine. Our earlier work has shown that manipulations in the expression of the key mediators of drug addiction in the NAc, such as the transcription factor ΔFosB, or the methyltransferase G9a, affect the development of analgesic tolerance (Zachariou *et al*, 2006; Sun *et al*, 2012). fMRI studies in chronic pain patients link changes in NAc activity to responses to noxious stimuli and suggest that the transition from acute to chronic pain involves changes in corticostriatal connectivity (Baliki *et al*, 2010, 2012). A more recent fMRI study in humans suggests that opiate analgesia is positively correlated with trait reward responsiveness and the response of the brain reward circuitry to noxious stimuli at baseline, before opiate administration (Waniqasera *et al*, 2012). Our earlier studies in *Rgs9* knockout mice revealed that this molecule modulates MOR function in an agonist-dependent manner (Psifogeorgou *et al*, 2011). Here, we show that changes in Rgs9-2 activity in the NAc of adult animals affect the analgesic responses to morphine and the development of morphine tolerance. These findings support our hypothesis that cellular mechanisms within the brain reward pathway affect analgesic responses to morphine. Optogenetic experiments helped us further understand the cellular mechanism of Rgs9-2 action in the NAc. Our behavioural findings implicate the D1-type neurons in the modulation of analgesic tolerance to morphine. For our optogenetic stimulation protocol, we selected a 10-Hz stimulation that has been used by several investigators (Lobo *et al*, 2010; Chandra *et al*, 2013) and selected stimulation durations that do not affect baseline Rgs9-2 levels or pain thresholds. Interestingly, activation of D1-type neurons in the presence of morphine leads to an increase in Rgs9-2 expression in the NAc. Rgs9-2 may form complexes with inhibitory as well as excitatory Gα subunits. It is therefore possible that Rgs9-2 modulates tolerance by blocking MOR signaling (as a negative modulator of MOR function) or by promoting D1/D5 signaling (by forming complexes with Gαq/Gαs subunits and acting as a positive modulator, Psifogeorgou *et al*, 2011). Thus, this first set of studies reveals that morphine dependence causes a robust downregulation of *Rgs9* mRNA and protein levels in D2-type neurons, whereas morphine tolerance is associated with increased Rgs9-2 activity in D1-type neurons. This is an important first step towards the understanding of the cell type- and morphine regimen-specific regulation of Rgs9-2 in the NAc. Future work will further determine the cell type-specific role of Rgs9-2 in dependence- and addiction-related behaviours, and the consequences of cell type-specific manipulations of Rgs9-2 activity in tolerance models.

While it is clear that several other spinal and supraspinal circuits modulate opiate analgesia and tolerance, our findings suggest that cellular adaptations in the NAc may influence analgesic responses to morphine. This is an important consideration for pain management, as chronic pain patients often develop opiate addiction (Ling *et al*, 2011). Information about the cellular mechanisms of opiate tolerance in the brain reward centre is also important for the development of more efficient pain management strategies for certain cases of severe chronic pain, where the development of addiction is not a major concern. In such cases, blockade of Rgs9-2 actions would permit the use of low morphine doses and would also delay the development of tolerance.

A major limitation of studies on Rgs9-2 function in the brain has been the lack of selective antibodies for immunofluorescence analysis, and the lack of pharmacological tools for *in vivo* interventions of Rgs9-2 activity. The AAV gene transfer system permits highly localized manipulations of Rgs9-2 activity in the brain of adult mice for a long period of time and provides an important tool for the study of NAc-specific adaptations following chronic morphine exposure. Our results provide novel important information about the function of Rgs9-2 in the brain reward pathway and point to Rgs9-2 complexes in the NAc as a potential target for the treatment of opiate addiction or the prevention of analgesic tolerance. Notably, our AAV microinjections covered both shell and core areas of the NAc; therefore, it is not possible to determine whether Rgs9-2 has distinct actions in these subregions. Our next set of studies will apply these tools to assess the way Rgs9-2 in the Nac modulates the reinforcing actions of morphine and other highly abused opiates. Given that Rgs9-2 is a negative modulator of the analgesic actions of morphine, blockade of Rgs9-2 activity in the NAc would permit the use of very low morphine doses to achieve an analgesic response, and would also be expected to delay tolerance; however, this manipulation would also increase the risk of addiction. Therefore, interventions in the function of Rgs9-2 complexes may be appropriate for conditions in which the risk of addiction is not a major concern. Current studies in our group are examining the function and regulation of Rgs9-2 complexes under chronic pain conditions. Future work should also determine the correlation between *RGS9* polymorphisms and substance abuse vulnerability or responsiveness to opiate analgesics. Finally, the development of compounds targeting Rgs9-2 interactions with Gβ5, R7BP, or Gα subunits will be an essential therapeutic tool as such interactions are the key for the stabilization and localization of Rgs9-2 (Terzi *et al*, 2009).

In summary, our study provides new information on the regulation of Rgs9-2 by chronic morphine, and the behavioural consequences of targeted Rgs9-2 overexpression in the NAc in various morphine actions. These findings enhance our understanding of the NAc signal transduction mechanisms mediating morphine addiction and analgesia.

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