Brain Region Specific Actions of Regulator of G Protein Signaling 4 Oppose Morphine Reward and Dependence but Promote Analgesia

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Background: Regulator of G protein signaling 4 (RGS4) is one of the smaller members of the RGS family of proteins, which are known to control signaling amplitude and duration via interactions with G protein α subunits or other signaling molecules. Earlier evidence suggests dynamic regulation of RGS4 levels in neuronal networks mediating actions of opiates and other drugs of abuse, but the consequences of RGS4 actions in vivo are largely unknown.

Methods: In this study, we use constitutive and nucleus accumbens-inducible RGS4 knockout mice as well as mice overexpressing RGS4 in the nucleus accumbens via viral mediated gene transfer, to examine the influence of RGS4 on behavioral responses to opiates. We also use electrophysiology and immunoprecipitation assays to further understand the mechanisms underlying the tissue-specific actions of RGS4.

Results: Inducible knockout or selective overexpression of RGS4 in the nucleus accumbens reveals that, in this brain region, RGS4 acts as a negative regulator of morphine reward, whereas in the locus coeruleus RGS4 opposes morphine physical dependence. In contrast, we show that RGS4 does not affect morphine analgesia or tolerance but is a positive modulator of certain opiate analgesics, such as methadone and fentanyl.

Conclusions: These findings provide fundamentally novel information concerning the role of RGS4 in the cellular mechanisms underlying the diverse actions of opiate drugs in the nervous system.

Key Words: AAV-Cre, analgesia, fentanyl, HSV-RGS4, locus coeruleus, morphine, nucleus accumbens, place preference, tolerance, withdrawal

Regulators of G protein signaling (RGS) are critical modulators of G protein-coupled receptor-mediated signal transduction via multiple interactions with G protein α subunits, scaffolds, and effector molecules (1–4). At least 10 of the 25 mammalian RGS proteins are expressed in the central nervous system (CNS) (5) and modulate essential physiological functions such as vision (6,7), locomotion (8), and working memory (9). In addition, several neuropsychiatric disorders including Parkinson's disease (10), addiction (11,12), and schizophrenia (13) are linked to dysfunctions of particular RGS proteins.

Regulator of G protein signaling 4 (RGS4), is a 28-kDa member of the R2 subfamily of RGS proteins, which is expressed widely in brain, including prefrontal cortex, striatum, locus coeruleus (LC), and hippocampus (5). The RGS4 consists of a 120-aa domain responsible for the GTPase-activating protein (GAP) activity that regulates G protein function and defines the RGS superfamily and an N-terminal element containing a cysteine-rich domain (N-end rule), which triggers arginylation and promotes ubiquitination and proteasomal degradation (14–16). A gene array analysis linked decreased levels of RGS4 in prefrontal cortex with schizophrenia and triggered a large number of clinical and preclinical studies on this subject (17). Most of these studies point to RGS4 as a vulnerability factor for schizophrenia (4,13,17–21), whereas evidence also supports a role of RGS4 in antipsychotic drug action (18,21,22). In striatum, RGS4 has a wide range of modulatory actions on muscarinic M2 autoreceptors (23) as well as on dopamine D1 and D2 receptors (4,24,25). Stress, corticosteroids, and drugs of abuse modulate RGS4 levels in several brain sites (11,26–28).

Previous work established the striatal enriched RGS9-2 as a key regulator of opioidergic and dopaminergic responses (8,12,29–32). The presence of RGS4 in striatal and areas mediating opiate actions and the evidence for the involvement of this RGS member in opiate physical dependence (4,11) led us to hypothesize that, in addition to RGS9-2, RGS4 might also be involved in opiate addiction. Opiates produce reward, physical dependence, and analgesia via activation of the G-protein coupled μ opioid receptor (MOR) (33,34). Although it is generally accepted that opiate addiction is associated with adaptations in MOR signal transduction, the cell-specific events remain incompletely understood (35–37). Here, we use constitutive and inducible knockout (KO) mouse models to examine the role of RGS4 in acute and chronic opiate actions. Our behavioral, electrophysiological, and biochemical findings establish that RGS4 exerts differential effects on distinct actions of opiates in the nervous system.

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Received Nov 20, 2008; revised Jul 1, 2009; accepted Aug 19, 2009.

Methods and Materials

Animals

Constitutive and inducible RGS4 mutant mice were generated as described in Supplemental Methods (Figure S1 in Supplement 1). All constitutive mutant mice used in this study were generated

0006-3223/10/$36.00
doi:10.1016/j.biopsych.2009.08.041

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from breedings of heterozygous RGS4 mice. For all behavioral assays, we used 2–3-month-old male KO mice and their wild-type (WT) littermates. For electrophysiological assays, we used 3–4-week-old male mice. For overexpression studies, 2–3-month-old C57/Bl6 mice were infected with herpes simplex virus (HSV)–β-galactosidase (LacZ) or HSV-RGS4 as described (12). For local KO of RGS4, 2–4-month-old floxed RGS4 male mice were bilaterally infected with adeno-associated virus (AAV)-Cre or AAV–green fluorescent protein (GFP). Animals were housed in a 12-hour dark/light cycle room according to the animal care and use committees of UT Southwestern Medical Center and the University of Crete. For place preference, analgesia, and opiate withdrawal paradigms, we used two-way analyses of variance and Bonferroni post hoc tests. For co-immunoprecipitation (IP) assays, we used one way analysis of variance and Dunnett's post hoc test. For western blotting we used *t* tests.

Behavioral Tests

A published unbiased conditioned place preference (CPP) procedure was used (8). For morphine locomotor activity assays, mice were placed in chambers as described (8), and ambulatory activity was monitored for 30 min after SC saline (days 1–3) or morphine (days 4–9) injections. Analgesia was measured with a 52°C hot plate apparatus (IITC Life Sciences, Woodland Hills, California), as described (12). For opiate withdrawal, mice received implantation with 25-mg morphine pellets; withdrawal was precipitated 3 days later with naloxone (1 mg/kg SC, Sigma, St. Louis, Missouri) as described (12). Fear conditioning was carried out according to published procedures (Supplement 1).

Laser Capturing and Polymerase Chain Reaction

Laser capture was performed as described (38). Floxed RGS4 mice received injection with either AAV-GFP or AAV-CreGFP into the nucleus accumbens (NAc) (39,40). Several weeks later, brains were coronally cryosectioned at 8 μm and mounted onto membrane slides (Leica, Deerfield, Illinois). Infected regions were laser-dissected and processed with PicoPure RNA extraction kit (Arcturus, Mountain View, California). The RNA was amplified with the RiboAmp kit (Arcturus) and reverse transcribed with superscript III (Invitrogen, Carlsbad, California). Quantitative polymerase chain reaction (qPCR) was performed as described previously (41) with SYBR Green (Applied Biosystems, Foster City, California) and primers for RGS4 (Fwd: GGCTGAATCGTTGGAAAACCT, Rvs: TGTTGCTTGCACTGAGATGAA) and glyceraldehyde-6-phosphate dehydrogenase (GAPDH) as a control.

Co-Immunoprecipitation, Western Blotting, and Viral Mediated Gene Transfer

Striatum from control or treated mice were rapidly dissected as described (42). The following antibodies were used for IP and western blotting: rabbit anti-MOR (Immunostar, Hudson, Wisconsin), rabbit anti-Gα_q (P. Sternweiss, UT Southwestern), a rabbit anti-RGS2 (provided by D. Siderovski, UNC, Chapel Hill), and rabbit anti-RGS4 (S. Mumby, UT Southwestern). For RGS4, in addition to a protease inhibitor cocktail (Sigma), samples contained a proteasome inhibitor (MG132, Sigma). The AAV-CreGFP (GFP-tagged Cre recombinase) and AAV-GFP vectors were produced with a triple transfection helper free method in human embryonic kidney (HEK) cells and purified, as described earlier (39,40). Stereotaxic surgery procedures are described in Supplement 1.

Electrophysiology

Recordings were obtained from LC neurons in brain slices obtained from drug-naive and morphine-dependent mice (treated as stated earlier). See Supplement 1 for a description of the protocols used, all of which are published (43–45). Recordings were made 2 hours after maintaining slices in a recording chamber to allow morphine to wash out fully from the slices (43).

Results

RGS4 and Morphine Reward: Actions in the Nucleus Accumbens

To assess the role of RGS4 in morphine reward, we induced a local KO of RGS4 in the NAc (part of the ventral striatum), a key brain reward region, of adult animals. This inducible, localized deletion of RGS4 was achieved by stereotaxic injection of an AAV vector expressing GFP-tagged Cre recombinase into the NAc of mice homozygous for a floxed RGS4 gene. Control animals received injection with AAV-GFP. Figures 1A and 1B show low- and high-power magnifications of AAV-CreGFP infected areas. To confirm recombination, we isolated the GFP-labeled region of the NAc by laser capture microdissection and measured RGS4 messenger RNA levels with qPCR. The RGS4 expression is reduced by >90% in the NAc of mice injected with AAV-CreGFP compared with mice injected with AAV-GFP (Figure 1C).

We next used CPP to determine how loss of RGS4 in the NAc affects morphine reward. The selective KO of RGS4 from this brain region increases sensitivity to the rewarding effects of morphine, as shown in Figure 1D, as the AAV-CreGFP injected mice exhibit a CPP at a 3 mg/kg dose of morphine, whereas AAV-GFP–injected control subjects require a higher morphine dose (5 mg/kg) to show a significant preference. A lower (1 mg/kg) morphine dose fails to establish a CPP in both groups. Sensitivity to morphine reward was also assessed with an overexpression model, where we injected an HSV vector expressing RGS4 (or LacZ as a control) into the NAc of WT C57Bl/6 mice. In contrast to the RGS4 local KOs, mice overexpressing RGS4 selectively in this brain region are significantly less sensitive to morphine (5 mg/kg SC) reward compared with LacZ expressing control subjects (Figure 1E). At higher morphine doses, both genotypes show the same CPP score (not shown). Together, these data support a role of RGS4 in the NAc in morphine reward.

In striking contrast with these local manipulations of RGS4 levels within the NAc, mice with constitutive KO of RGS4 do not exhibit morphine CPP even at high drug doses (CPP score in seconds for WT animals: 1 mg/kg morphine = 29 ± 45 mg/kg, 5 mg/kg morphine = 143 ± 32; for RGS4 KO mice: 1 mg/kg morphine = 46 ± 105 mg/kg, 5 mg/kg morphine = 4.0 ± 52). This finding likely reflects loss of RGS4 from other brain regions where it is expressed or at early times during development and emphasizes the importance of using inducible and brain region-specific KO strategies.

To further assess the role of RGS4 in the NAc in regulating behavioral responses to morphine, we tested mice with local RGS4 KOs from this brain region and AAV-GFP–injected control mice, in a locomotor sensitization paradigm. Consistent with the CPP data, deletion of RGS4 from NAc accelerates the development of locomotor sensitization to repeated morphine exposure (Figure 1F).

RGS4 and Morphine Dependence: Actions in the LC

We next examined the role of RGS4 in morphine physical dependence. Constitutive RGS4 KO mice were implanted with

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