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RGS9-2–controlled adaptations in the striatum determine the onset of action and efficacy of antidepressants in neuropathic pain states

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Edited by Robert C. Malenka, Stanford University School of Medicine, Stanford, CA, and approved July 24, 2015 (received for review March 3, 2015)

The striatal protein Regulator of G-protein signaling 9-2 (RGS9-2) plays a key modulatory role in opioid, monoamine, and other G-protein–coupled receptor responses. Here, we use the murine spared-nerve injury model of neuropathic pain to investigate the mechanism by which RGS9-2 in the nucleus accumbens (NAc), a brain region involved in mood, reward, and motivation, modulates the actions of tricyclic antidepressants (TCAs). Prevention of RGS9-2 action in the NAc increases the efficacy of the TCA desipramine and dramatically accelerates its onset of action. By controlling the activation of effector molecules by G protein α and βγ subunits, RGS9-2 affects several protein interactions, phosphoprotein levels, and the function of the epigenetic modifier histone deacetylase 5, which are important for TCA responsiveness. Furthermore, information from RNA-sequencing analysis reveals that RGS9-2 in the NAc affects the expression of many genes known to be involved in nociception, analgesia, and antidepressant drug actions. Our findings provide novel information on NAc-specific cellular mechanisms that mediate the actions of TCAs in neuropathic pain states.

Regulator of G-protein signaling 9-2 (RGS9-2) is an intracellular modulator of G-protein–coupled receptor (GPCR) function, which is expressed in medium spiny neurons and cholinergic interneurons of the striatum (1, 2). RGS9-2 influences the magnitude and time course of GPCR signaling by promoting GTPase activity of the Go subunit and by preventing activation of Go effectors (3). This modulation can also influence the duration of interactions between the Gβγ subunits and their effector molecules. In addition to Go subunits, RGS9-2 interacts with several scaffolds and signal transduction proteins that affect its function, expression, and cellular localization. Interactions with the Gβγ subunit and the adaptor protein R7BP determine the stability and cellular localization of RGS9-2, respectively (3, 4). Several recent studies have provided information on the regulation and function of RGS9-2 complexes in the striatum and how these complexes affect pharmacologic responses (2, 5, 6). In particular, RGS9-2 has been shown to modulate the actions of various psychotropic, antiparkinsonian, neuroleptic, and opiate analgesic drugs (1, 7). The nucleus accumbens (NAc) is a striatal brain region that is a major site of antidepressant drug action (8). Recent studies provided information on signal transduction events triggered by tricyclic antidepressants (TCAs) in NAc neurons and identified several second messengers, transcription factors, and epigenetic molecules involved in their therapeutic actions (9–11). TCAs, such as desipramine (DMI) and nortriptyline (NTL), and selective serotonin/norepinephrine reuptake inhibitors (SNRIs) have also been used to treat neuropathic pain, a complex chronic disorder that is highly comorbid with anxiety and depression (12, 13) and is characterized by thermal hyperalgesia, mechanical allodynia, and dysesthesia (14). TCAs act mainly as serotonin/norepinephrine transporter inhibitors, but additional actions on other GPCRs or ion channels may also contribute to their therapeutic effect (15, 16). In neuropathic pain models, TCAs modulate monoaminergic responses at the spinal cord level, but less is known about their actions in supraspinal sites.

Here, we use genetically modified mice, viral-mediated gene transfer, and several biochemical and molecular biology assays to probe the mechanism via which RGS9-2 in the NAc modulates TCA responses under neuropathic pain states (17, 18). We use the murine spared-nerve injury (SNI) model of neuropathic pain, which produces several symptoms of neuropathic pain, including mechanical allodynia, thermal hyperalgesia, anxiety, and depression (17–19). The results indicate that RGS9-2 plays a prominent role in the onset and efficacy of TCAs with regard to the antiallodynic and antidepressant effects. RGS9-2 negatively regulates the activity of several protein kinase A (PKA) targets, as well as complexes between Gβγ subunits and the GPCR kinase 2 (GRK2) and histone deacetylase 5 (HDACS). Furthermore, our findings indicate that ablation of Rgs9 prevents the nuclear shuttling and gene-silencing effects of HDACS observed during the initial stages of DMI treatment that contribute to the delayed onset of action. RNA-sequencing (RNA-seq) analyses revealed that RGS9-2 affects the expression of a variety of genes involved in TCA responses and/or nociceptive behaviors. Together, these findings provide information on the cellular actions of TCAs in the NAc.

Significance

Neuropathic pain is a complex disorder, characterized by affective and sensory symptoms. Efficient treatment of this condition should target both pain-modulating pathways and mood/affect networks. We show that tricyclic antidepressants (TCAs), which modulate spinal pain processing, also act in the brain reward center to alleviate allodynia and depression-like behaviors. We reveal how one key protein of nucleus accumbens (NAc)-specific signaling affects several molecules/pathways with emerging roles in antinociceptive and antidepressant mechanisms. Our study provides information about the cellular adaptations induced by TCAs in the NAc and novel targets for pain treatment.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE71527).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1504283112/-/DCSupplemental.
under neuropathic pain states and identify several factors that affect TCA onset of action and therapeutic efficacy.

Results

Our earlier work demonstrated that RGS9-2 modulates depression-like behaviors developed at 2 mo after nerve injury, but does not affect the intensity of mechanical allodynia or thermal hyperalgesia (19). Here, we use the murine SNI model to test for a role of RGS9-2 in the antiallodynic and antidepressant actions of TCAs. As shown in Fig. 1A, Rgs9−/−-wild-type (Rgs9WT) and Rgs9-knockout (Rgs9KO) mice underwent SNI surgery and began treatment with DMI 2 wk later (a time when maximal allodynia has been established in both genotypes). Mechanical allodynia was evaluated by using the Von Frey test at several time points before and during DMI treatment. In agreement with our earlier findings (19), baseline mechanical allodynia was indistinguishable between Rgs9KO and Rgs9WT mice. Nevertheless, 10 d into a chronic low-dose DMI treatment (5 mg/kg, i.p., twice a day), Von Frey responses were significantly increased in Rgs9KO mice (Fig. 1B). SNI or DMI treatment did not affect the locomotor activity of Rgs9WT or Rgs9KO mice (Fig. 1I). Administration of the TCA NTL (3 mg/kg, i.p., twice a day; Fig. 1C) produced an antiallodynic response in Rgs9KO mice only at 2 wk of treatment. The SNRI duloxetine (DLX; 5 mg/kg, i.p., twice a day; Fig. 1D) produced a similar phenotype: Rgs9KO–SNI mice showed an earlier antiallodynic response in the Von Frey test compared to their wild-type controls. An additional cohort of mice was used to determine whether Rgs9KO mice show earlier antiallodynic responses when a high dose of DMI is administered (15 mg/kg, i.p., twice a day). As shown in Fig. 1E, both genotypes showed attenuation of allodynia; however, Rgs9KO mice responded much earlier than Rgs9WT mice. Together, these data suggest that prevention of RGS9-2 action shifts the DMI dose–response curve to the left and also accelerates the onset of the antiallodynic effect. To evaluate the role of RGS9-2 in the antidepressant efficacy of DMI, SNI mice were further treated with DMI (10 mg/kg, i.p., 24 h; 10 mg/kg, i.p., 5 h; and 20 mg/kg, s.c., 1 h before testing) after the 2-wk DMI treatment and monitoring of Von Frey responses and then evaluated in the forced swim test (FST). As shown in Fig. 1F, Rgs9KO–SNI mice that showed increased sensitivity to the antiallodynic actions of DMI also showed enhanced sensitivity to DMI in the FST. Baseline FST immobility time is not different between genotypes because Rgs9KO mice develop depression-like behavior at 8 wk after SNI (19) and FST assays here are performed at 5 wk after SNI.

To investigate the contribution of RGS9-2 in the NAc to the antiallodynic effects of DMI, we used a viral-mediated gene-transfer approach. Rgs9WT and Rgs9KO mice were treated with a low dose of DMI (5 mg/kg, i.p., twice a day) starting at 2 wk after SNI, and on day 23 they were stereotaxically injected with herpes simplex viruses (HSVs) expressing Rgs9-2 or control β-galactosidase (LacZ) (2). DMI administration resumed 1 d after surgery. The HSV viruses were selected because of their rapid onset of action (peak expression at 3 d after stereotaxic infection). Animals were divided into three groups: Rgs9KO–HSV–Rgs9-2, Rgs9KO–HSV–LacZ, and Rgs9WT–HSV–LacZ. As shown in Fig. 2A, restoration of RGS9-2 expression in the NAc of Rgs9KO mice rescues the DMI phenotype observed in the Von Frey assay. A separate series of gene transfer studies were performed for NAc-specific Rgs9-2 overexpression, by using a longer-lasting adeno-associated virus (AAV) gene-transfer method. This time, mice were infected with AAV–Rgs9-2 or AAV–GFP (control) constructs, and SNI surgery was performed 2 wk after infection, when the viral expression is maximal (5). Although Rgs9-2 overexpression in the NAc does not affect baseline mechanical allodynia responses, it prevents the antiallodynic actions of a standard DMI regimen (10 mg/kg, i.p., twice a day for 2 wk; Fig. 2B).

Coimmunoprecipitation (co-IP) assays were performed next, to determine whether DMI treatment (10 mg/kg, i.p., twice a day) affects the association of RGS9-2 with its interacting partner Gβ5, an effect known to promote RGS9-2 stability. After 2 wk of DMI treatment (a time point at which C57BL/6 mice have not developed an antiallodynic response), there was an increase in complexes between RGS9-2 and control βγ subunits (Fig. 3A). Administration of the SNRI duloxetine (5 mg/kg, i.p., twice a day) affects the association of RGS9-2 with its interacting partners Gα and Gβγ subunits (Fig. 3A). Therefore, preventing RGS9-2 activity in the NAc would lead to an earlier onset of DMI action and increase drug efficacy via enhanced Gα or Gβγ signaling. To this end, we treated Rgs9WT and Rgs9KO mice with DMI and collected NAc tissue 2 wk after DMI treatment (15 mg/kg, i.p., twice a day), a time point when Rgs9KO, but not wild-type, mice show an antiallodynic response. Our first set of Western blot analyses monitored
the effect of DMI on PKA target proteins. As shown in Fig. 3C and D, DMI treatment leads to a decrease in glutamate receptor subunit 1 (GluR1) (S845) and cAMP response element-binding protein (CREB) (S133) phosphorylation sites without affecting total protein levels.

We next investigated the effect of DMI on Gβγ-related signal-transduction events. GPCR activation is known to promote the formation of complexes between Gβγ subunits and GRK kinases (20, 21). We hypothesized that the antiallodynic response observed with low doses of DMI in Rgs9KO mice is associated with increased levels of GRK2/Gβ complexes, and we used co-IP assays to monitor these complexes in ventral striatal tissue. As shown in Fig. 3E, DMI treatment promotes the formation of complexes between GRK2 and Gβ subunits in the Rgs9KO group. GPCR activation also promotes complexes between Gβ subunits and the epigenetic modifier HDAC5 (22, 23). HDAC5 is expressed in several brain regions, mediating sensory and affective responses and TCA actions (10, 24). Here we show that chronic DMI promotes complexes between Gβ subunits and a phosphorylated (cytoplasmic) form of HDAC5 (S498), whereas these complexes are markedly decreased in the NAc of Rgs9KO mice (Fig. 3F). HDAC5 can be found in both cytoplasmic and nuclear compartments, and the nuclear translocation of this protein is controlled by phosphorylation (25, 26), as well as by complexes with Gβγ subunits (22, 23). Because DMI promotes complexes between Gβ subunits and HDAC5, we hypothesized that upon DMI administration, HDAC5 translocates to the nucleus and binds to chromatin complexes to suppress gene expression (an effect that delays the onset of drug action). When we used a nuclear/cytoplasmic fractionation protocol and monitored HDAC5 and Gβ levels in nuclear and cytoplasmic compartments, we found that acute DMI application (15 mg/kg, i.p.) increases the levels of both HDAC5 and Gβ proteins in the nucleus without affecting the nuclear/cytoplasmic distribution of Gβ5 and RGS9-2 (Fig. 4A). The acute DMI stimulation of HDAC5 nuclear translocation was absent in Rgs9KO mice (Fig. 4B). We also found that after chronic DMI administration (3 wk, a time point when both genotypes respond to DMI), no nuclear translocation of HDAC5 is observed in Rgs9WT–SNI or Rgs9KO–SNI mice (Fig. 4C). Notably, at the 2-wk time point at
which only Rgs9KO animals show a response to DMI, no changes in HDAC5 phosphorylation at site Ser-498 were observed, further supporting the hypothesis that there is no nuclear translocation under these conditions (Fig. S2).

We then investigated the role of HDAC5 in antiallodynic responses to DMI. Genetic ablation of Hdac5 shifted the DMI dose–response to the left, because Hdac5 knockout mice, but not their wild-type littermates, showed antiallodynic responses to a low-dose regimen of DMI (5 mg/kg, twice a day; Fig. 4D). To determine whether HDAC5 actions in the NAc contribute to this phenotype, we used HSV-mediated gene transfer to overexpress Hdac5 in adult C57BL/6 mice after 2 wk of SNI and 5 d of DMI treatment. As shown in Fig. 4E, HSV–Hdac5 infection in the NAc prevented the antiallodynic effect of DMI (10 mg/kg, twice a day) in the Von Frey test.

The first set of our studies demonstrated that ablation of the Rgs9 gene affects several protein–protein interactions, phosphorylation events, and epigenetic mechanisms that are associated with DMI responses. Because the therapeutic effects of TCAs are associated with changes in gene expression (8, 27), the last part of this study used RNA-seq analysis to gain an understanding of the adaptations in gene expression associated with knockout of Rgs9, nerve injury, and DMI treatment in the NAc. For the RNA-seq studies, we used six different groups of mice: naive Rgs9WT and Rgs9KO, SNI-operated Rgs9WT and Rgs9KO mice treated with saline, and SNI-operated Rgs9WT and Rgs9KO mice treated with DMI. We used similar time points of DMI treatment (15 mg/kg, i.p., twice a day) as in our behavioral assays. NAc tissue from wild-type and mutant mice was collected at the time point of 2 wk, when only knockout mice show an antiallodynic response (Figs. 1E and 5A). Results were analyzed as pairwise...
comparisons between the groups, and gene regulation is inferred by fold change. Information regarding gene ontology (GO) and number of genes in each experimental group is provided in Fig. S3. To further illustrate a role of Rgs9 on gene adaptations in the NAc, we constructed Venn diagrams of data from wild-type and mutant mice compared with their corresponding SNI–DMI groups (Fig. 5B). As expected, the impact of drug treatment was evident on the Rgs9KO SNI–DMI mice, because this group demonstrates an antiallodynic response at the time point examined. However, the changes between saline-treated and naive Rgs9KO mice were almost one-third of those observed in the wild-type groups, although neuropathic pain affects the expression of a large number of genes in the NAc of wild-type mice (Fig. 5C). Even the 10 overlapping genes changed in the opposite direction, as illustrated in the embedded heatmap and in Table S1. Interestingly, when including the differences in gene adaptations between the naive wild-type and knockout groups, we found that more than half of the genes affected by Rgs9 deletion overlap with the genes altered after nerve injury induction in Rgs9WT mice, suggesting that naive Rgs9KO mice possess adaptive changes in the expression of genes involved in neuropathic pain.

Next, we used heatmap analysis to depict the significant fold changes in gene expression between Rgs9WT and Rgs9KO mice, from the naive, SNI–Sal-, and SNI–DMI-treated groups. Using as reference the significantly changed genes from the comparison of naive mice groups, the heatmap reveals that gene regulation pattern in the NAc of Rgs9WT mice is very similar to the gene regulation induced by DMI treatment (Fig. 5D). Conversely, when genes from the SNI–DMI condition were used as
reference, the resulting heatmap produced a very similar pattern, suggesting that DMI treatment reverses the effect of nerve injury on gene expression, which now resembles gene regulation in naive animals (Fig. S4A). Ontology analysis revealed that both naive and SNI–DMI groups are involved in similar cellular processes and pathways (Fig. S4B). Annotation of the genes from these groups to specific categories using keyword terms shows that most of the genes are related to signal transduction events (Fig. S5A). Moreover, 53 of the genes overlap with those changed between the naive and DMI-treated SNI groups of Rgs9WT and Rgs9KO mice (Fig. 5E). Representative genes are included in the table in Fig. 5E. Specifically, in Rgs9KO NAc, the early response to DMI induces the expression of genes encoding ion channels, transcription factors, neuronal guidance/differentiation, and signal transduction molecules known to be involved in several forms of pain and/or antidepressant drug actions. For example, mice lacking the adenylate cyclase-activating polypeptide 1 (Adcyap1) do not develop inflammatory or neuropathic pain and exhibit depression-like behaviors (28, 57). We show a reduction of Adcyap1 expression in the NAc of Rgs9KO mice that respond to DMI treatment, supporting the hypothesis that reduction of ADCYAP1 activity prevents pain-like

Fig. 5. NAc-specific RNA-seq profiling of Rgs9 knockout in naive and neuropathic pain mice, treated either with saline or DMI. (A) Timeline of the RNA-seq experiments. Briefly, NAc tissue was collected at the time point when only the mutant mice responded to the chronic DMI treatment. (B) Venn diagram depiction of the effect of drug treatment on gene expression. As expected, gene expression was slightly affected in the NAc of Rgs9WT–DMI mice compared with saline-treated controls, because tissue collection was performed at the time point at which only the mutant mice developed antiallodynic response. (C) Neurogenic pain effect on gene expression in drug-naive mice. Venn diagram between the groups of naive and neuropathic pain-treated Rgs9WT and Rgs9KO mice suggests that the effect of SNI on gene expression was less potent in the NAc of mutant mice, whereas SNI affected different genes in the wild-type vs. mutant mice. The small embedded heatmap shows that even most of the 10 overlapping genes change to opposite direction. When differentially expressed genes between naive Rgs9WT and Rgs9KO mice were added to the Venn diagram, it was revealed that half of the adaptations induced after SNI surgery in wild-type mice are already present in the NAc of the naive mutant mice, possibly explaining the small effect in the Rgs9KO NAc after SNI. (D) Heatmap analysis reveals that the gene regulation pattern in the NAc of Rgs9KO naive mice is very similar to the gene regulation induced by DMI treatment, suggesting that Rgs9-specific gene adaptations in the absence of Rgs9 promote DMI’s actions. (E, Left) Venn diagram of the genes from naive and SNI–DMI groups of Rgs9WT and Rgs9KO mice reveals an overlap of 53 genes with very similar regulation, as indicated by the embedded heatmap. (E, Right) The table lists some of these genes with the corresponding fold changes. Importantly, the direction of the change is similar between the groups of naive and SNI–DMI-treated mice, suggesting that this adaptive gene regulation in the Rgs9KO naive mice facilitates DMI’s antiallodynic effect.
responses (Fig. 5E). Moreover, the negative regulation of
brain-derived neurotrophic factor (Bdnf) in the Rgs9KO SNI–
DMI group correlates with reports on a role of Bdnf in
neuropathic pain (29). Notably, the frizzled-B (FzB) and
dickkopf-like 1 (Dkk1) genes (Fig. 5E) are members of the Wnt
signaling pathway, which has an emerging role in neuropathic
pain pathogenesis, depression, and antidepressant drug actions
(11, 30).

Heatmaps of the genes between group comparisons of
Rgs9KO mice are shown in Fig. 6A. Chronic DMI treatment
completely reverses the gene signature established after SNI
induction in the knockout mice. This reveals that the majority of
these genes regulate cell adhesion, neuronal differentiation, and
cell motion (Fig. S6). The table in Fig. 6B lists several of these
genes, which are implicated in nociception, depression, and an-
tidepressant drug actions. For example, the transcription factors
Bhlhe22 and Npas4 have been implicated in nociceptive and
depressive behaviors respectively (58, 59). Importantly, the di-
rection of gene expression change in the SNI–DMI-treated
Rgs9KO mice is the same as in the naive Rgs9KO mice, sug-

gesting that the basal gene expression profile upon knockout of
Rgs9 facilitates DMI actions. Some of the genes altered by DMI
treatment in Rgs9KO mice with SNI encode proteins that are
predicted via the STRING database to interact with each other
(Fig. 6C). RNA-seq analysis findings were verified by quantita-
tive PCR (qPCR). Fig. 6D and E shows data from qPCR analysis
of NAc cDNA from naive Rgs9WT and Rgs9KO mice. Changes
in expression of mRNAs between Rgs9WT and Rgs9KO SNI

Fig. 6. Rgs9 gene knockout promotes adaptive changes that resemble DMI's therapeutic effect. (A) Heatmap of the genes regulated by chronic DMI in the NAc of Rgs9KO SNI group reveals that chronic antidepressant treatment almost completely reversed the adaptive changes in the group of mutant mice under
neuropathic pain, suggesting that ablation of Rgs9 promotes adaptive changes that facilitate DMI's actions. (B) Table depicting fold changes for the genes between the aforementioned group comparison as well as the naive group of mice. Importantly, the direction of the change for many genes is similar
between the first and the third group comparisons shown in the table, suggesting once again that adaptive changes in the NAc of Rgs9KO naive mice are
beneficial for the action of DMI. (C) Moreover, by using the STRING database for protein–protein interactions, a highly interconnected network of gene
products from the Rgs9KO SNI–DMI compared with Rgs9KO SNI–Sal group was revealed. (D and E) To validate the RNA-seq results, several genes from the
groups of naive Rgs9WT vs. Rgs9KO and Rgs9WT SNI–DMI vs. Rgs9KO SNI–DMI mice were selected to be tested with qPCR. (D) Up-regulated genes. (E) Down-
regulated genes. *P < 0.05; **P < 0.01; ***P < 0.001 (t test, unpaired two-tailed). (F) Similarly, 18 of 22 selected genes (19 genes shown here) from the group
of mice that responded to DMI treatment were confirmed by qPCR (data expressed as log2 fold change). *P < 0.05 (t test, unpaired two-tailed). N.c.,
no change.
mice after 2 wk of DMI treatment are shown in Fig. 6F. Adenylate cyclase 1 (Adcy1), along with the Adcyap1, Bdnf, and Cholecystokinin (Cck) genes, are part of protein-interaction networks that form between products of the genes belonging to the comparison of SNI–DMI groups of mice as shown in Fig. S5B.

Discussion

Neuropathic pain is a complex chronic disorder that involves adaptations in the spinal cord, as well as in several brain regions controlling pain perception, mood, motivation, and learning. Recent reports implicate multiple forebrain regions, such as the amygdala, the anterior cingulate cortex, and the NAc, in the pathophysiology of chronic pain syndromes (31–33). The data presented here demonstrate that RGS9-2 complexes in the NAc negatively modulate a number of cytoplasmic and nuclear events, which ultimately regulate the onset of action and therapeutic efficacy of DMI and other antidepressants in a neuropathic pain state. Although it is clear that alleviation of chronic pain symptoms by TCAs involves adaptations in monoamine responses in several CNS regions, the data presented here indicate that modulation of GPCR signaling in the NAc by RGS9-2 contributes to their therapeutic effectiveness.

We focused on the investigation of RGS9-2 because this molecule is a potent modulator of GPCR activity in the striatum and affects behavioral responses to several psychoactive or monoamine-targeting compounds (2, 5). Several preclinical studies have shown that DMI has antiallodynic properties in models of neuropathic pain (34–37). The DMI dose–response may vary between genetic backgrounds, neuropathic pain models, and type of Von Frey hair, but overall, chronic DMI administration promotes antiallodynic responses in rodents. DMI treatment affects the activation of the transcription factor CREB and is associated with changes in phosphoprotein levels in the brain reward center (9, 18, 38, 39). By controlling the availability of Gα and βγ subunits to effector molecules, RGS9-2 negatively regulates protein interactions and levels of phosphoproteins involved in antidepressant responsiveness. Here, we show that chronic DMI administration reduces CREB (S133) and GluR1 (S845) phosphorylation in the NAc of Rgs9KO mice. Both of these phosphorylation sites are targets of PKA (40). Notably, a recent study demonstrated that this GluR1 phosphorylation in the insular cortex contributes to postsynaptic amplification of neuropathic pain (33).

We also show that ablation of Rgs9 affects the activation of Gβγ effectors such as GRK2 and HDAC5. Acute DMI administration promotes the formation of complexes between HDAC5 and Gβδ subunits and transiently promotes nuclear translocation of HDAC5, which disappears with prolonged treatment concurrent with the manifestation of the antiallodynic response. The lack of DMI-induced HDAC5 nuclear translocation in Rgs9KO mice likely explains the accelerated antiallodynic phenotype observed in these mice. Consistent with these data, the onset and efficacy of DMI antiallodynic action are also altered by Hdac5 deletion. Because DMI treatment enhances complex formation between GRK2 and HDAC5, future work should elucidate the Gβδ subunit specificity of these events, as well as the role of other HDACs in the NAc in DMI responsiveness. Nevertheless, these data suggest that compounds targeting HDAC5 may amplify effects of TCAs in the treatment of neuropathic pain. However, manipulation of RGS9-2 activity may be more advantageous than targeting HDAC5, because RGS9-2 is primarily expressed in the striatum. Although no pharmacologic tools for manipulating RGS9-2 activity are currently available, several efforts are directed toward targeting key protein interactions to antagonize the effects of RGS9-2 complexes.

We also used a more open-ended approach to identify the genes in the NAc regulated by DMI under neuropathic pain states. RNA-seq analyses reveal that chronic DMI alters expression of a number of genes, including those encoding GPCRs, ion channels, adenylyl cyclase targets, and components of the Wnt signaling pathway—several of which have been shown to play a prominent role in nociceptive transmission, mood disorders, and antidepressant drug actions. Differential gene-expression analysis revealed the down-regulation of many genes with DMI treatment after SNI in Rgs9KO mice compared with wild-type controls, including Adcyap1 and Bdnf, for which reduced function is associated with reduced neuropathic pain sensitivity (28, 29), and Adcy1, the deletion of which reduces mechanical allodynia and inflammatory pain sensitivity (41). The expression of regulator of neurite outgrowth neuritin 1 (Nrn1), a gene that is stimulated by Bdnf and is implicated in antidepressant actions (42, 43) and diabetic neuropathy (44), is also altered. Several other genes with documented roles in chronic pain and antidepressant actions that are down-regulated by chronic DMI treatment in the Rgs9KO SNI group, including Neuropeptide Y1 receptor (Npy1r) (Fig. 6B, 60), were also altered (45). Neuropeptide systems constitute major drug development targets for the treatment of anxiety and depression, which are often comorbid with chronic pain (46, 47). Our RNA-seq study also reveals that DMI down-regulates Cck and vasointestinal peptide (VIP) in Rgs9KO mice, similar to what has been reported with chronic imipramine treatment (43). Interestingly, Cck in mediobasal–prefrontal–NAc projections mediates depressive symptoms, and blockade of the Cck B receptor promotes resilience (48). Notably, the pattern of Cck and Vip regulation is similar between naïve Rgs9KO and wild-type mice, suggesting that adaptive changes in the NAc of Rgs9KO mice establish a pattern/signature that facilitates the antiallodynic effect of DMI. Cck and Vip, as well as Adcyap1, have been proposed as promising therapeutic targets for the treatment of neuropathic pain (49) and have been listed in other published microarray datasets from stress and depression studies (10, 43, 50) (Table S2). Also regulated by DMI in Rgs9KO mice were the peptides oxytocin, which has been implicated in antinoceptive mechanisms (51), and galanin (Galan), which has a profound role in nerve injury (52, 53) and neuropathic pain–induced motivational changes (54).

Our RNA-seq studies also identified a number of GPCRs known to be involved in antidepressant drug actions and chronic pain. This list includes the gene encoding dopamine receptor D1 (Drd1a), which is potently regulated by RGS9-2 (Fig. 6E). This receptor positively modulates nociceptive responses (55), and its expression is decreased in the NAc 28 d after the induction of nerve injury (56). Moreover, chronic TCA treatment leads to an increase in Drd1a expression in the rodent NAc (43). Our findings suggest that the up-regulation of Drd1a expression in the NAc of naïve Rgs9KO mice facilitates the actions of DMI. Finally, our results support a major role of the Wnt signaling pathway in DMI responses (Fig. 6E and Figs. S3A and S4B). This pathway contributes to the development of hyperalgesia and allodynia after nerve injury (30) and is involved in antidepressant drug actions (43).

In summary, this study provides information on NAc-specific intracellular mechanisms modulating the antiallodynic and antidepressant actions of TCAs in neuropathic pain states. RGS9-2 modulates a wide range of cytoplasmic and nuclear functions that both enhance DMI efficacy and accelerate the onset of action. RGS9-2 acts via a complex mechanism involving protein interactions, phosphorylation events, and control of HDAC5 repressor function. Our work provides additional evidence on the interrelationship between chronic pain and depression, showing that neuropathic pain promotes adaptations in networks associated with mood and motivation several weeks before the development of mood disorders. Pharmacological interventions that target some of these adaptations in the NAc not only reverse depression-like behaviors but also alleviate sensory deficits such as mechanical allodynia, most likely by altering the perception of
noxious stimuli. As the NAc emerges as a key regulatory region for both sensory and affective symptoms of neuropathic pain, future studies will use information from proteomic analysis and the RNA-seq studies to further understand the GPCRs and pathways affected by RGS9-2 to identify novel drug targets. We will also evaluate the role of RGS9-2 in the therapeutic actions of other categories of monoamine targeting antidepressants that are used for the treatment of chronic pain (such as venlafaxine and tapentadol) to determine the role of specific monoamine receptors in drug responsiveness as well as the role of specific Gβ subunits and epigenetic molecules. We now aim to develop novel pharmacologic strategies to target RGS9-2 complexes to accelerate the onset of action and to enhance the therapeutic efficacy of antidepressants in models of chronic pain and depression.

Materials and Methods

Animals and Drug Treatments. For all behavioral experiments, 2- to 3-mo-old Rgs9 or Hda5 knockout mice and their wild-type littermates or adult C57BL/6 mice (The Jackson Laboratory) were used. All behavioral studies were performed in adult male mice, except for the NTL and DLX Von Frey studies, which were performed in adult female mice. Mice were group-housed (four or five per cage) on a 12-h light/dark cycle, provided with food and water ad libitum. Animal handling was in accordance to the animal care and use committees of Icahn School of Medicine at Mount Sinai and the University of Crete. DMI (Sigma Aldrich), NTL (Sigma Aldrich), and DLX (Tocris Bioscience) were dissolved in distilled water and further diluted in saline. Saline was also used as vehicle treatment. Injections were performed at 9:00 AM and at 9:00 PM. More details about drug injections for each of the provided experiments are included in Results.

Stereotoxic Surgery and Local Viral Localization. Stereotoxic coordinates for viral vector injections into the NAc were as follows: AP=+1.6, AL=+1.5, and DV=−4.4 at an angle of 10° from the midline (S). HSV–Rgs9-2, HSV–Hda5, and HSV–LacZ vectors were generated by R.L.N. (2, 10). AAV p-HDAC5 (S498; 1:1,000; Abcam), rabbit anti-β-tubulin (1:40,000; Sigma-Aldrich), and tapentadol (1:20,000; W. Simonds, National Institute of Diabetes, Digestive and Kidney Diseases, Bethesda), rabbit anti-Gji (1:40,000; Santa Cruz), rabbit anti-p–HDAC5 (S498; 1:1,000; Abcam), rabbit anti–p–CREB (S133; 1:1,000; Cell Signaling), rabbit anti-CREB (1:1,000; Cell Signaling), rabbit anti–p–GLUR1 (S845; 1:1,000; Cell Signaling), rabbit anti–GLUR1 (1:1,000; Cell Signaling), mouse anti–HDAC5 (1:1,000; Abcam), mouse anti–β-actin (1:10,000; Sigma-Aldrich), and mouse anti–GRK2 (1:2,000; Santa Cruz). Rabbit anti–p–HDAC5 (S279; 1:1,000; ref. 24) was provided by C. W. Cowan (Harvard University).

RNA-Seq Studies and qPCR. Six groups of mice with three biological replicates per group were used for the RNA-seq study. In brief, NAC punches were taken from individual mice (six animals per treatment group), and total RNA was isolated from pooled bilateral punches of two animals per replicate (three replicates per treatment group), as described above with TRIzol according to manufacturer’s protocol. mRNA was purified with RNasey Micro columns (Qiagen), and an Agilent 2100 Bioanalyzer confirmed that the RNA integrity numbers were >8.0. The poly-A-containing mRNA was purified by using poly-T oligo-attached magnetic beads, and the mRNA-seq library was prepared from each pooled RNA sample by using the TruSeq RNA Sample Preparation Kit v2 (no. RS-122-2002), according to the instructions of Illumina. RNA-seq was performed on the Illumina HiSeq2000 machine at Mount Sinai’s Genomic Core facility. The RNA-seq read alignment and differential analysis were performed by using the TopHat2 (62) and Cufflinks (63) packages. Significantly differentially expressed genes were determined by using a false discovery rate of <5% in this analysis. Details on RNA-seq quality control metrics are provided in Table S3. qPCR was performed by using SYBR green (Quanta Biosciences; catalog no. 95073) on an Applied Biosystems 7500 system. Reactions were run in triplicate and analyzed by using the ΔΔCT method and GAPDH as normalization control.

Bioinformatic Analysis. To identify pathway and gene functional annotations that were overrepresented in our RNA-seq results, GO analysis was performed by using the functional annotation and clustering software tool available through the online database for annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources (Version 6.7, https://david.ncifcrf.gov/). Three GO terms, biological process (BP), gene function (MF), and Swis sProt and Protein Information Resource (SP) keywords were used to cluster genes. Only terms with P < 0.05 are reported. The Venn diagrams were generated by using the BioVenn-web application (www.cmbi.ru.nl/cdb/biovenn/index.php). To identify known and potential protein interactions between the products of the genes involved in our RNA-seq results, we used the STRING (Version 9.1) software and database (64).

Statistical Analysis. For all behavioral experiments, data were analyzed by using two-way ANOVA followed by the Bonferroni post hoc test, as indicated in each figure. For Western blot and co-IP assays, we used the Student’s t test.

ACKNOWLEDGMENTS. This work was supported by National Institute on Drug Abuse Grant PPG-PO1DA08227 (to V.Z.), National Institute of Neurological Disorders and Stroke Grant NS086444 (to V.Z. and L.S.), and the Greek Secretariat for Research and Technology and the 7th EU Framework (Aristia I; to V.Z., D.T., V.M., and S.G.).

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