

Galanin negatively modulates opiate withdrawal via galanin receptor 1

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Abstract

Rationale The neuropeptide galanin has been shown to modulate opiate dependence and withdrawal. These effects could be mediated via activation of one or more of the three distinct G protein-coupled receptors, namely galanin receptors 1 (GalR1), 2 (GalR2), and 3 (GalR3).

Objectives In this study, we used several transgenic mouse lines to further define the mechanisms underlying the role played by galanin and its receptors in the modulation of morphine dependence. First, transgenic mice expressing β -galactosidase under the control of the galanin promoter were used to assess the regulation of galanin expression in response to chronic morphine administration and withdrawal. Next, the behavioral responses to chronic morphine administration and withdrawal were tested in mice that over-express galanin, lack the GalR1 gene, or lack the GalR2 gene.

Methods Transgenic and matched wild-type mice were given increasing doses of morphine followed by precipitation of withdrawal by naloxone and behavioral responses to withdrawal were assessed.

Results Both morphine administration and withdrawal increased galanin gene transcription in the locus coeruleus (LC). Increasing galanin levels in the brain reduced signs of opiate withdrawal. Mice lacking GalR1 undergo more severe opiate withdrawal, whereas mice lacking GalR2 show no significant difference in withdrawal signs, compare with matched wild-type controls.

Conclusions Opiate administration and withdrawal increase galanin expression in the LC. Galanin opposes the actions of morphine which leads to opiate dependence and withdrawal, an effect that is mediated via GalR1.

Keywords Galanin · Galanin receptor 1 · Mouse · Opiate · Addiction · Withdrawal · Locus coeruleus

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Abbreviations

LacZ	β -Galactosidase
GalR1	Galanin receptor 1
GalR2	Galanin receptor 2
GalR3	Galanin receptor 3
LC	Locus coeruleus
WT	Wild type

Introduction

The neuropeptide galanin is widely expressed in the hypothalamic–pituitary–adrenal axis and in the central

nervous system, including areas of the limbic system and regions of the brain that are important for mediating reward, motivational, dependence, and withdrawal responses to drugs of abuse, such as the hypothalamus, hippocampus, and locus coeruleus (LC), (Melandar et al. 1986; Koob and Volkow 2010). Consistent with its distribution in these brain areas, increasing evidence demonstrates that galanin plays an important role in modulating the neurochemical and behavioral correlates of addiction (Picciotto 2008; Picciotto et al. 2010).

The physiological effects of galanin are mediated by the activation of one or more of three distinct G protein-coupled receptors, namely galanin receptors 1 (GalR1), 2 (GalR2), and 3 (GalR3) (Burgevin et al. 1995; Kolakowski et al. 1998; Wang et al. 1997). Galanin binding sites are present in areas of the rat brain important for mediating the effects of drugs of abuse (Skofitsch et al. 1986) and GalR1 and GalR2, and to a lesser extent, GalR3 mRNAs, have distinct but overlapping distributions in the limbic system (Mennicken et al. 2002; O'Donnell et al. 1999).

Several studies have shown a role for galanin in drug reward-related behaviors. Administration of galanin into the lateral ventricles attenuates the development of conditioned place preference for morphine (Zachariou et al. 1999), and galanin knockout mice are more sensitive to the locomotor stimulant properties of morphine and show increased morphine-conditioned place preference compared to matched wild-type (WT) mice (Hawes et al. 2008). Galanin also modulates the behavioral response to psychostimulants such as amphetamine and cocaine (Brabant et al. 2010; Kuteeva et al. 2005; Narasimhaiah et al. 2009). Galanin also appears to modulate opiate dependence and withdrawal. The neuropeptide is expressed in most of the noradrenergic neurons of the LC (Xu et al. 1998), and expression is increased after chronic morphine administration and withdrawal (McClung et al. 2005). In vitro studies have shown that galanin decreases the firing of LC neurons by hyperpolarizing LC noradrenergic neurons (Mennicken et al. 2002; Rasmussen et al. 1990; Seutin et al. 1989; Sevcik et al. 1993), an effect hypothesized to decrease signs of opiate withdrawal. In vivo administration of the small molecule galanin receptor agonist, galnon, alleviates morphine withdrawal symptoms. Furthermore, behavioral signs of withdrawal are exacerbated in galanin knockout mice and attenuated in mice which over-express galanin in NA neurons using the dopamine β -hydroxylase promoter (Zachariou et al. 2003).

Here, we use several genetically modified mouse lines to further investigate the mechanism underlying the ability of galanin to modulate morphine physical dependence and the expression of opiate withdrawal. In the absence of galanin receptor selective compounds that can be administered systemically, these lines provide a useful tool for under-

standing the mechanisms through which galanin mediates its actions. The current study provides evidence that galanin opposes morphine dependence and withdrawal via a mechanism that involves activation of GalR1 receptors. Thus, GalR1 receptor-specific agonists may provide new pharmacological treatments for opiate dependence and withdrawal.

Methods

Animals All animals were fed standard chow and water ad libitum and housed under identical conditions. Principles of laboratory animal care were followed, and animal care and procedures were performed within the UK Home Office and University of Crete protocols and guidelines. Adult (~10 week old) male mice (~25 g) were used in each experiment with strain, sex and age-matched WTs, derived from the same colony (see below), as controls. Mouse lines were generated as follows:

Galanin-LacZ mice Details of the transgene construction, generation, strain, and breeding history of these mice have been described previously (Bacon et al. 2007). Briefly, galanin-LacZ mice (Gal-LacZ mice) were generated using a ~25-kb transgene containing the entire murine galanin genomic locus with ~20 kb of the upstream sequence, and a 3.5-kb *LacZ* gene inserted into the 5'-UTR 13 bp downstream of the transcriptional start site. The transgene has been maintained on the CBA X C57BL6F1 hybrid background. Expression of transgene-derived β -galactosidase (*LacZ*) recapitulates the expression pattern of endogenous galanin.

Galanin overexpressing mice Details of the generation, strain, and breeding history have been described previously (Bacon et al. 2007; Elliott-Hunt et al. 2004). In brief, galanin overexpressing mice (GalOE mice) were generated using a ~25-kb transgene containing the entire murine galanin coding region and 19.9 kb of upstream sequence. Transgenic lines were bred to homozygosity and have been maintained on the CBA X C57BL6F1 hybrid background. The transgenic line denoted OE46 used in this study has high levels of galanin expression in the brain (see (Bacon et al. 2007), for further details).

Galanin receptor 1 mutant mice GalR1 knock out mice were generated as described previously (Jacoby et al. 2002). Briefly, a targeting vector containing the majority of exon 1 of the murine *GalR1* gene was used to transfect the W9.5 ES cell line, in order to generate mice that carry an insertional inactivating mutation within the first exon of the GalR1 gene. Mice remain inbred on the 129T2/SvEmsJ strain.

Galanin receptor 2 mutant mice Details of the generation, strain, and breeding history of GalR2 knock out mice have been described previously (Hobson et al. 2006). In brief, mice deficient for the *GalR2* gene were generated by and licensed from Lexicon Genetics. The 5.17-kb gene trap vector VICTR48 (Viral Construct for TRapping) was inserted within the single intron of the murine *GalR2* gene in a 129Sv/EvBrd ES cell line clone (Zambrowicz et al. 2003). Omnibank clone OST105469 was used to obtain germ-line transmission of the disrupted *GalR2* allele. Heterozygote pairs on the 129/SvEvBrd X C57BL6 background were then bred to homozygosity and maintained on that background.

Transgenic or mutant founders were bred with WT of the same strain to generate heterozygous mice. Heterozygous mice were then bred to generate homozygous WT and transgenic or mutant mice. Subsequent generations of mice were obtained from homozygous pairings.

Opiate withdrawal For opiate withdrawal assays, mice were injected with increasing morphine doses (from 20 to 100 mg/kg), every 8 h for 3 days, and withdrawal was precipitated using naloxone hydrochloride (1 mg/kg, subcutaneously (s.c.), Sigma), 3 h after the last morphine injection. Withdrawal signs (jumps, wet dog shakes, tremor, ptosis, paw tremor, mastication, backward walking, diarrhea, and weight loss) were monitored for 30 min after naloxone administration (Zachariou et al. 2003). The global withdrawal score was calculated based on the following formula: (number of backward walking steps \times 0.1)+(number of jumps \times 0.1)+(ptosis)+(tremor)+(weight loss in percent)+(diarrhea)+(number of wet dog shakes).

Locomotor activity assay Mice were habituated to handling for 3 days prior to locomotor testing. On test days, mice were placed in the testing room for 30 min of habituation. Locomotion was measured in 20 \times 20 cm boxes from Med Associates (ENV-256C Med Associates, Inc, St. Albans, VT, USA). For saline response sessions, mice were injected s.c. immediately before being confined to the chamber for 30 min. For morphine response sessions, mice were injected with morphine (3 or 5 mg/kg) s.c. immediately before being confined to the chamber for 30 min to determine early phase locomotor activity. Movement of each mouse was measured by photocell beam breaks and was recorded with Med-PC IV software. One saline and one morphine session were carried out each day for 3 days; the three locomotor session values for each mouse were averaged and normalized to saline response session values in WT mice.

Immunohistochemistry For immunohistochemical studies, mice were perfused with 4% paraformaldehyde as described previously (Zachariou et al. 2003). Briefly,

40- μ m coronal brain sections were washed in phosphate-buffered saline (PBS) (pH 7.5), incubated in blocking buffer (3% Normal Donkey Serum, 0.3% Triton X-100) for 1 h and with rabbit β -gal primary antiserum (1:8,000, Chemicon AB1211) overnight. The next day, slices were washed 3 times in PBS, incubated with Cy2 anti-rabbit IgG (1:400 Jackson, PA, USA), washed 3 times with PBS, mounted, dehydrated, and cover-slipped. Images were acquired with a Zeiss microscope. Numbers of LacZ-positive nuclei were averaged from the left and right side of LC sections from each animal. All sections were quantified on the same day under the same conditions by an experimenter blind to the treatment groups.

Results

To investigate the effect of chronic morphine treatment and withdrawal on the modulation of galanin expression in the CNS, we used transgenic mice expressing LacZ under the control of a 20.5-kb fragment of the galanin promoter (Bacon et al. 2007). This allowed us to study the effects of morphine on galanin promoter activation. LacZ expression was examined in three groups of animals: the first group was treated with saline and the other two groups of animals received increasing doses of morphine. Three hours after the last treatment, animals received an injection of naloxone (NLX) or saline. The three groups were (a) saline-NLX, (b) morphine-saline, and (c) morphine-NLX. As shown in Fig. 1b, repeated morphine administration results in an increase in LacZ expression in the LC in Gal-LacZ mice. Figure 1c shows that LacZ expression in the LC is further enhanced following withdrawal. As shown in Fig. 1d, there was a $283\pm 75\%$ increase in the number of LacZ-positive nuclei following opiate withdrawal, compared to control animals. Morphine produces a small but not significant decrease in the number of LacZ nuclei in the LC. The observed activation of the galanin promoter by morphine and morphine withdrawal is selective, since no change in LacZ expression was observed in the hippocampus (not shown).

We next examined the consequences of galanin overexpression on the behavioral signs of morphine dependence and withdrawal. We used a previously characterized galanin overexpressing transgenic line (OE46) in which high levels of galanin were observed in many brain regions. GalOE mice and their matched WT controls were evaluated in the opiate withdrawal paradigm. As shown in Fig. 2 and consistent with our earlier findings (Bacon et al. 2007; Zachariou et al. 2003), galanin overexpression leads to a milder withdrawal syndrome, as several withdrawal signs, i.e., jumps, wet dog shakes, and backward

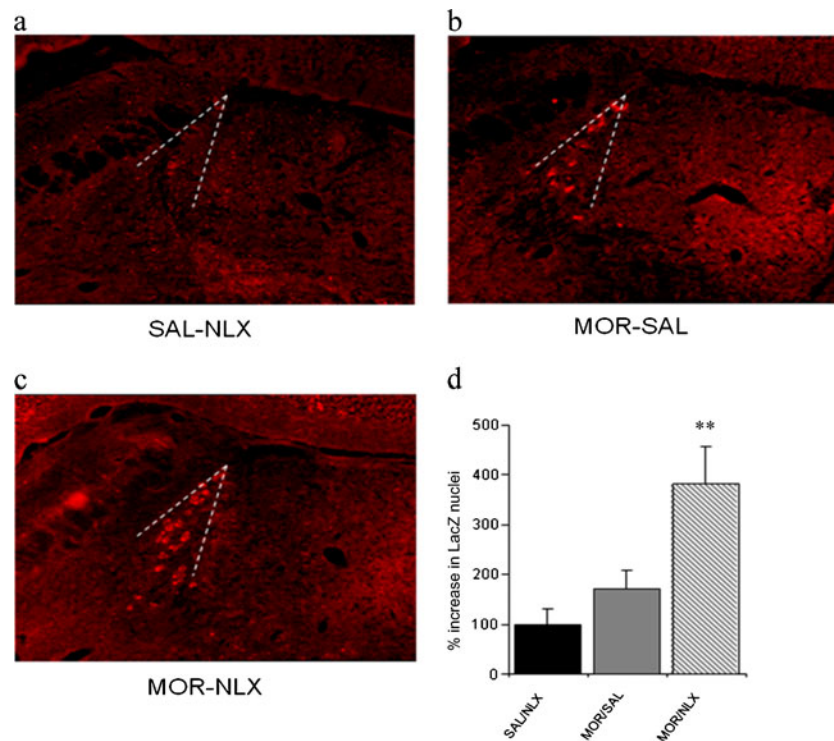


Fig. 1 Regulation of galanin promoter activity by chronic morphine and morphine withdrawal. Transgenic mice expressing beta-galactosidase (LacZ) under the control of the galanin promoter were injected with increasing doses of morphine for 3 days, as described in the “Methods” section, and withdrawal was precipitated using naloxone. Animals received saline (a) or morphine (b, c) and on the third day received a saline (b) or naloxone (a, c) injection 3 h after the last morphine injection to precipitate withdrawal. Quantitation of the

LacZ-positive neurons in the LC following saline-naloxone (SAL-NLX), morphine (MOR), and morphine-naloxone (MOR-NLX) treatment (d), there was a significant increase in the number of LacZ-positive nuclei after morphine-naloxone treatment ($n=4-5$ per group, $**p<0.01$, one way ANOVA followed by Dunnett post hoc test). LacZ expression in the LC was monitored using immunofluorescence. Micrographs were taken at $\times 20$

walking are significantly reduced. The global withdrawal score is 26 ± 3 for WT controls and 18.3 ± 2 for GalOE ($p<0.05$, t test).

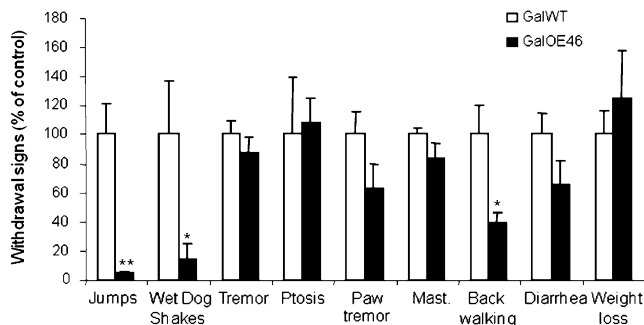


Fig. 2 Galanin overexpression in the locus coeruleus leads to a milder withdrawal syndrome. Mice overexpressing galanin (GalOE) and their wild-type (WT) controls (derived from the homozygous offspring of heterozygous breeding pairs) received increasing doses of morphine for 3 days, as described in the “Methods” section, and withdrawal was precipitated using naloxone. Withdrawal signs (Jumps, wet dog shakes, tremor, ptosis, paw tremor, mastication (Mast.), backward walking, diarrhea, and weight loss) were monitored for 30 min after naloxone injection. Data are expressed as mean \pm SEM ($n=8$ per group). $*p<0.05$, $**p<0.01$, two way ANOVA followed by Bonferroni correction for multiple comparisons

The last part of the study sought to determine which galanin receptor subtype mediates the actions of galanin on morphine dependence. As both GalR1 and GalR2, and to a lesser extent GalR3, are expressed in the LC, we used mice lacking a functional GalR1 (galanin receptor 1 mutant (GalR1 mut)) or GalR2 (galanin receptor 2 mutant (GalR2 mut)) gene and their respective matched WT controls in the morphine withdrawal paradigm. To date a GalR3 knockout has not been described. Our findings indicate that mice lacking GalR1 exhibit more severe withdrawal behavior compared to their matched WT controls. In particular, GalR1 mut mice show significantly increased jumps, tremor, paw tremor, ptosis, and diarrhea (Fig. 3; Global withdrawal scores for GalR1 WT= 21.7 ± 2 and for GalR1 mut= 30.3 ± 1 , $p<0.05$, t test).

Analysis of GalR2 mutant mice in the same behavioral paradigm indicates that this receptor subtype is not critical for opiate withdrawal, as there is no difference between GalR2 mutants and matched WTs in morphine withdrawal behavioral symptoms. Global withdrawal score for GalR2 WT= 18.4 ± 1 and for GalR2 mut= 16.6 ± 2.3 (Fig. 4).

Notably, GalR1 and GalR2 mutant mice show similar locomotor activation to their matched WT controls after

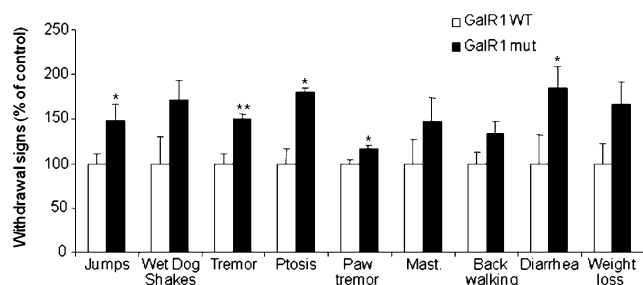


Fig. 3 GalR1 modulates morphine withdrawal behaviors. GalR1 mutant mice (*GalR1 mut*) and their wild-type (*WT*) controls (derived from the homozygous offspring of heterozygous breeding pairs) were injected with increasing doses of morphine for 3 days, as described in the “Methods” section, and withdrawal was precipitated using naloxone. Withdrawal signs were monitored for 30 min after naloxone injection. Knockout of the GalR1 gene significantly exacerbated several signs of morphine withdrawal, including jumps, tremor, ptosis, paw tremor, and diarrhea. Data are expressed as mean±SEM ($n=7$ per group). * $p<0.05$, ** $p<0.01$, two way ANOVA followed by Bonferroni correction for multiple comparisons

morphine administration (Fig. 5), at least during the initial 30-min phase of locomotor activity. However, these results do not preclude a potential influence of GalR1 or GalR2 on later morphine-induced locomotor activity.

Discussion

The neuropeptide galanin and its receptors are widely expressed in the brain and are present in networks known to be important for development and expression of opiate withdrawal (Melander et al. 1986; O'Donnell et al. 1999). GalR1, GalR2, and GalR3 couple to $G_{i/o}$ inhibit adenylyl cyclase and decrease cAMP levels, and activate the GIRK family of potassium channels (Habert-Ortoli et al. 1994;

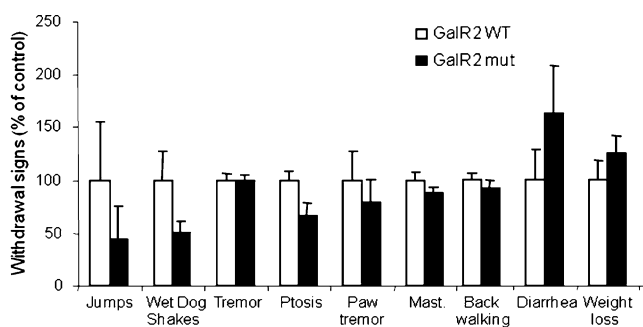


Fig. 4 Absence of GalR2 does not affect opiate withdrawal behavior. GalR2 mutant mice (*GalR2 mut*) and their wild-type controls (*GalR2 WT*) (derived from the homozygous offspring of heterozygous breeding pairs) were injected with increasing doses of morphine for 3 days, as described in the “Methods” section, and withdrawal was precipitated using naloxone. Withdrawal signs were monitored for 30 min after naloxone injection. Lack of the GalR2 gene had no significant effect on morphine withdrawal signs. Data are expressed as mean±SEM ($n=8$ per group)

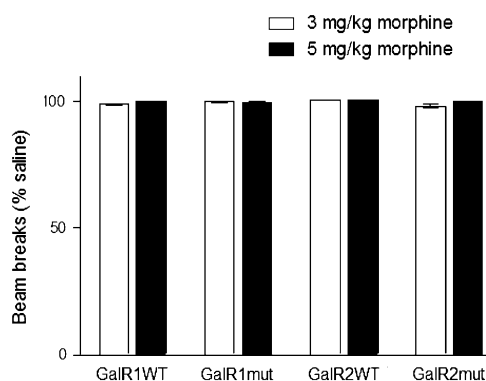


Fig. 5 Absence of GalR1 or GalR2 does not affect the initial locomotor activating actions of morphine. GalR1 and GalR2 mutant mice (*GalR1 mut*, *GalR2 mut*) and their respective matched wild-type controls (*WT*) were injected with low morphine doses and their locomotion was measured over 30 min. All mice showed comparable beam breaks regardless of genotype. Data are expressed as a percent of average saline beam breaks±SEM

Smith et al. 1998). GalR2 also signals via $G_{q/11}$ to activate phospholipase C and protein kinase C (Branchek et al. 2000).

Galanin can modulate neuronal activity in brain regions related to drug dependence and withdrawal. The neuropeptide is expressed in most of the noradrenergic neurons of the LC (Xu et al. 1998) and is increased after chronic morphine administration and withdrawal (McClung et al. 2005). Studies in vitro have shown that galanin decreases the firing of LC neurons by hyperpolarizing LC noradrenergic neurons (Rasmussen et al. 1990; Seutin et al. 1989; Sevcik et al. 1993), an effect hypothesized to decrease signs of opiate withdrawal. This has been proposed to be due to a GalR1-mediated increase in membrane permeability to potassium ions (Pieribone et al. 1995).

The current dataset demonstrates that galanin expression is regulated in the LC under conditions leading to morphine dependence and withdrawal, and shows that GalR1 is critical for mediating the effects of galanin to reduce opiate withdrawal signs. Therefore, these studies identify GalR1 as a novel target for the treatment of opiate dependence and withdrawal. This study complements earlier work on the ability of the galanin system to oppose the behavioral correlates of morphine dependence and withdrawal (Zachariou et al. 2003).

Opiate dependence and withdrawal produce adaptive changes in the cAMP pathway, and one of the most robust changes is increased adenylyl cyclase levels, and thus cAMP production, in the noradrenergic LC neurons (Kogan et al. 1992). In vitro studies show that galanin inhibits cAMP formation following chronic morphine administration and withdrawal (Hawes et al. 2006). The first evidence for a potential role of the GalR1 subtype in the actions of galanin on opiate withdrawal came from in

situ hybridization studies showing upregulation of this receptor in LC neurons following chronic morphine administration and withdrawal (Zachariou et al. 2000). Furthermore, GalR1 expression in the LC is also modulated by galanin signaling. Both the opiate administration/withdrawal-induced upregulation of GalR1 and the modulation of GalR1 levels by galanin occur through a cAMP-dependent mechanism involving phosphorylation of the transcription factor cAMP regulatory element-binding protein that regulates the GalR1 promoter (Zachariou et al. 2001; Hawes et al. 2005). Here, we show that transcription of the galanin peptide is also increased in LC neurons following opiate dependence and withdrawal. Consistent with the opponent process theory (Koob and Le 2008; Koob and Volkow 2010), this upregulation, along with an increase in GalR1, may be part of a compensatory mechanism to oppose both the increased firing and cAMP production of the LC neurons following chronic morphine administration and withdrawal (Hawes et al. 2005; Zachariou et al. 2000). Consistent with the previous findings that application of the galanin agonist galanin alleviates several signs of opiate withdrawal (Zachariou et al. 2003), we show that galanin overexpression in the LC leads to a milder withdrawal syndrome, most likely mediated via GalR1. Interestingly, in naïve animals, galanin appears to have a different effect on stress-mediated responses, as galanin overexpression leads to augmented stress-evoked noradrenaline release (Kehr et al. 2001). We speculate that galanin has distinct roles in stress responses in naïve versus morphine-dependent mice. Also, as mentioned above, galanin mu-opioid receptor interactions in the LC are critical for the modulation of LC firing (Sevcik et al. 1993). It is possible that GalR mu-opioid receptor dimers can be formed and might have distinct signaling properties that would be abolished in GalR1 knockout mice, resulting in increased LC firing in response to noradrenaline, and a more severe withdrawal phenotype, but this remains to be explored at the molecular level.

Our behavioral data using genetically modified mice, lacking GalR1 or GalR2, show that GalR1 is selectively involved in regulating opiate withdrawal behaviors, as mice lacking this receptor subtype show significantly more exaggerated symptoms of morphine withdrawal than their WT controls, whereas the GalR2 knockout mice show no overall significant withdrawal phenotype. However, although not statistically significant, there is some reduction in jumps, wet dog shakes, and ptosis, which might be attributed to compensatory changes in GalR1 receptor expression/function in GalR2 mutants. These behavioral studies were conducted on several genetically modified mouse lines and their respective WT controls. Transgenic or mutant founder animals were paired with WTs of the same strain to generate heterozygotes, from which were bred homozygous transgenic

or mutant mice and their respective WTs. Therefore, although these studies did not use littermates derived from continuous heterozygous breeding (which can be a limitation when interpreting behavioral data, but avoids the generation of unwanted mice), the WTs used were strain, age and sex matched, and housed in an identical environment. Our findings are in agreement with previous knowledge and are coherent between the various genetically modified mice used in this study. These studies help clarify the function of galanin receptor subtypes in morphine dependence and withdrawal and provide important information for new pharmacological strategies for the treatment of morphine dependence and addiction.

Several lines of evidence including *in situ* hybridization, gene array analysis, pharmacological, and behavioral studies indicate an important role for the galanin peptide and GalR1 receptor subtype in opiate reward, dependence, and withdrawal (Picciotto 2008). In spite of the importance of alleviating withdrawal to help addicts achieve abstinence, very few drugs to date are available to treat opiate withdrawal symptoms. Compounds targeting $\alpha 2$ adrenergic receptors, such as clonidine, show limited success and have significant side effects. Thus, GalR1 agonists may provide a novel approach to alleviate opiate withdrawal. In summary, the current set of studies provides novel information about the mechanisms underlying the actions of galanin in opiate dependence and withdrawal and point to GalR1 as a potential new pharmacological target.

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