

Upregulation of Galanin Binding Sites and GalR1 mRNA Levels in the Mouse Locus Coeruleus Following Chronic Morphine Treatments and Precipitated Morphine Withdrawal

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The neuropeptide galanin and its receptors are expressed in the locus coeruleus (LC), a brain area associated with drug dependence and withdrawal. Although galanin peptide mRNA levels do not change during withdrawal, it is not known whether galanin receptor levels are regulated following opiate withdrawal. This study demonstrates that galanin binding in the LC is upregulated by chronic-intermittent morphine administration or by precipitated withdrawal, but not by acute morphine treatment, suggesting that increased activity in the LC may be able to regulate galanin binding sites. Moreover, the increase in

galanin binding sites seems to be caused by increased transcription or stabilization of the galanin receptor 1 (GalR1) gene, because there is a dramatic increase in mRNA levels following withdrawal in the LC. It is, therefore, possible that the increase in GalR1 could be an adaptive mechanism that leads to regulation of cAMP levels and possibly firing rate of LC neurons.

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The neuropeptide galanin (Melandar et al. 1986b) and galanin binding sites (Melandar et al. 1986a; Skofitsch et al. 1986) are present in most norepinephrine containing neurons of the locus coeruleus (LC), a brain area that plays a role in opiate dependence and the expression of somatic withdrawal symptoms (Aghajanian 1978; Nes-

tlar 1992). In vitro studies have shown that activation of galanin receptors inhibits the spontaneous firing of LC neurons (Pieribone et al. 1995; Seutin et al. 1989; Sevcik et al. 1993). However, intracerebroventricular (ICV) galanin fails to suppress somatic opiate withdrawal symptoms at doses that will induce feeding (Holmes et al. 1994), and mRNA levels of the galanin peptide were unchanged following morphine withdrawal (Holmes et al. 1995b). One reason galanin fails to ameliorate withdrawal symptoms could be that the galaninergic system is maximally activated under these conditions and that compensatory changes during withdrawal occur at the level of the receptor rather than the peptide.

Activation of galanin receptors leads to reduction of intracellular calcium levels via different mechanisms, depending upon the tissue. In the brain, there is evi-

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dence that the inhibitory effect of galanin is mediated via inhibition of adenylate cyclase (Nishibori et al. 1988). Adenylate cyclase activity, as well as G-protein and cAMP levels, are known to be increased in the LC during opiate dependence and withdrawal, and adaptive changes in receptors negatively linked to these effectors might be part of a regulatory mechanism to reduce the activity of these neurons. Three galanin receptor subtypes have been cloned to date: GalR1, GalR2, and GalR3 (Habert-Ortoli et al. 1994; Wang et al. 1997a; Wang et al. 1997b). GalR1 is found in many brain areas, including the ventral hippocampus, olfactory tract, hypothalamus, nucleus accumbens (nAc), and LC. GalR2 is found in the dentate gyrus, cingulate nucleus, posterior hypothalamic, supraoptic and arcuate nuclei as well as in the periphery (Kolakowski et al. 1998). GalR3 is very abundant in the periphery but is also present in the olfactory cortex, the hippocampal CA regions, and the dentate gyrus (Kolakowski et al. 1998; Wang et al. 1997b). Based on the distribution of galanin receptors, GalR1 is likely to comprise most galanin binding sites in the LC.

The goal of this study is to examine changes in galanin receptor binding following morphine treatment and withdrawal. Furthermore, we have examined the mRNA encoding GalR1 following morphine treatment to determine whether changes in galanin binding are attributable to increased transcription of this gene. Our hypothesis is that regulation of galaninergic transmission by opioids may occur at the level of the galanin receptor, rather than the peptide, and that functional interaction between these systems may occur in brain areas responsible for drug dependence and with-

drawal. Therefore, we performed receptor autoradiography and in situ hybridization studies in animals that were either chronically treated with morphine, or in animals that had undergone naltrexone-precipitated withdrawal, determine how galanin binding sites and GalR1 mRNA are regulated by these treatments.

MATERIALS AND METHODS

Animals

Female C57Bl/6 mice weighing 22–24 g (Jackson Laboratories, Bar Harbor, ME) were housed in a temperature and humidity controlled vivarium, provided food and water ad libitum, and were maintained on a 12 h dark/light schedule. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Drug Treatments

Acute Morphine. Morphine (100 mg/kg) or saline were injected SC ($n = 5$ per group). Mice were sacrificed by decapitation 5 h after treatment, and brains were dissected and frozen at -80°C .

Repeated Morphine. Morphine or saline were administered SC at 10 mg/kg once a day for 10 days ($n = 5$ per group). Mice were sacrificed 30 min or 48 h following the last treatment, and brains were frozen at -80°C .

Chronic Morphine. Mice were injected IP with increasing doses of morphine every 8 h for 2 days (20, 40, 60, 80, 100, 100, 100 mg/kg morphine) following a pro-

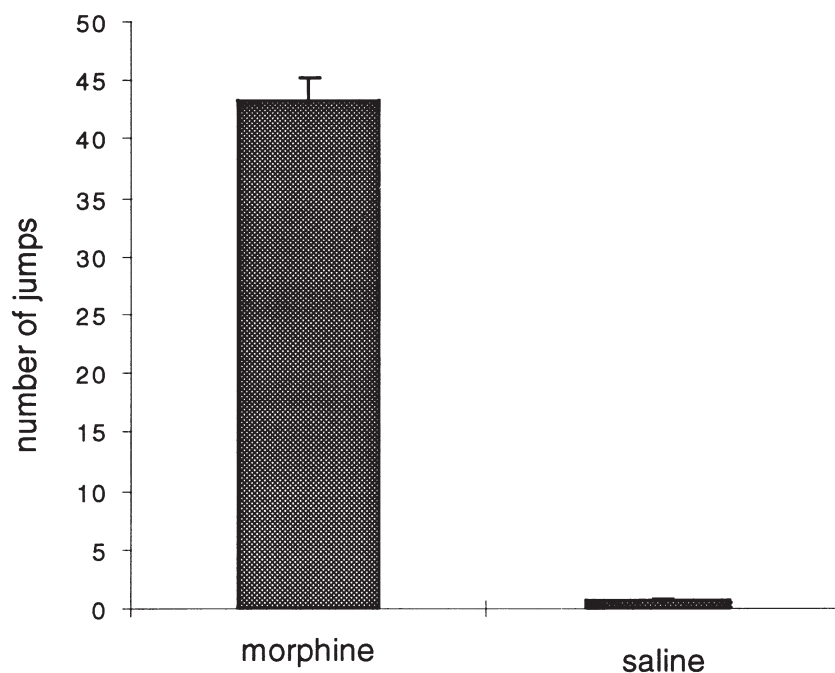


Figure 1. Jumping behavior as a sign of somatic withdrawal in mice. Jumping behavior was observed for 30 min following injection of 1 mg naltrexone (SC). Mice were either treated with increasing doses of morphine (20, 40, 60, 80, 100, 100, 100 mg/kg) or with saline every 8 h for 2.5 days ($n = 8$ for each group). All animals in the morphine-treated group exhibited repeated jumping after naltrexone administration; whereas, only two saline-treated animals showed isolated jumps, and none exhibited repeated jumping. Data are presented as number of jumps \pm SEM $*p < .05$ as compared to saline controls.

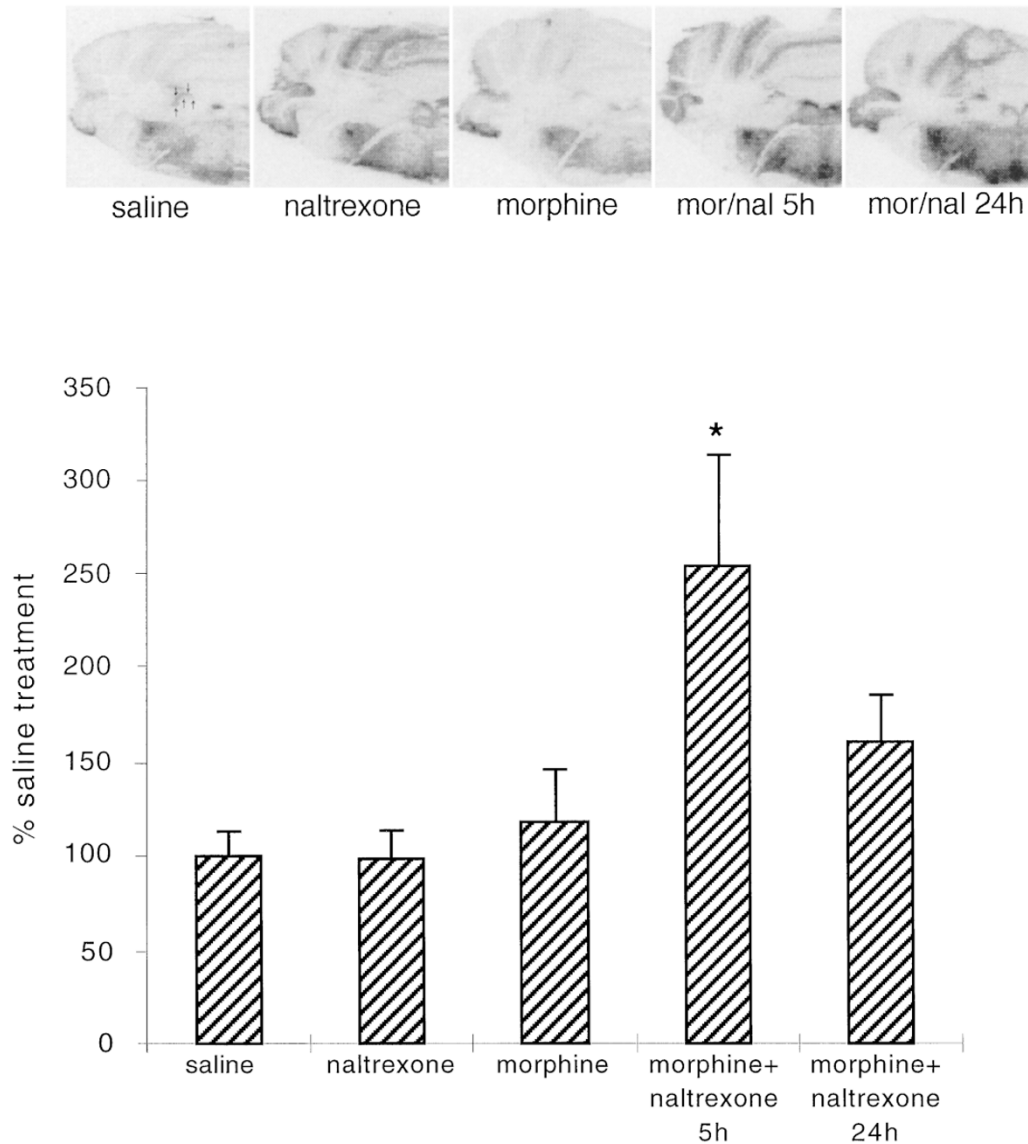


Figure 2. Changes in [125 I] galanin binding in the LC following saline ($n = 10$), naltrexone ($n = 9$), chronic morphine ($n = 10$), and two time points (5 and 24 h) following naltrexone precipitated withdrawal ($n = 10$). Galanin binding was significantly increased in the LC 5 h following naltrexone-precipitated withdrawal and recovered close to baseline at 24 h. Area quantitated is indicated by small arrows in saline-treated section. * $p < .05$ as compared to saline controls.

tolcol that has been used to induce morphine withdrawal in C57Bl/6 mice (Maldonado et al. 1996). Two h following the final morphine injection, mice received 1 mg/kg naltrexone SC. Animals were sacrificed 5 or 24 h after naltrexone injection for binding studies and 3 h after naltrexone injection for in situ hybridization studies. This protocol represents a mild withdrawal protocol, but significant signs of withdrawal (jumping, ptosis, chewing, diarrhea, and weight loss) were observed within 3 h following naltrexone injection. Five groups of mice were used for binding studies. First, mice that received chronic morphine were sacrificed 24 h after acute naltrexone injection ($n = 10$). Second, mice that re-

ceived chronic morphine were sacrificed 5 h after acute naltrexone injection ($n = 10$). Third, mice that received chronic morphine, an acute saline injection instead of naltrexone, were sacrificed 5 h after saline injection ($n = 10$). Fourth, mice that received chronic saline were sacrificed 24 h after acute naltrexone injection ($n = 9$). Fifth, mice that received chronic saline, an acute saline injection instead of naltrexone, were sacrificed 24 h after the last injection ($n = 10$). Time points for sacrifice following treatment were selected based on the assumption that at least 3 h would be required to detect changes in surface receptor levels and that most changes should be detected within 24 h. All brains were

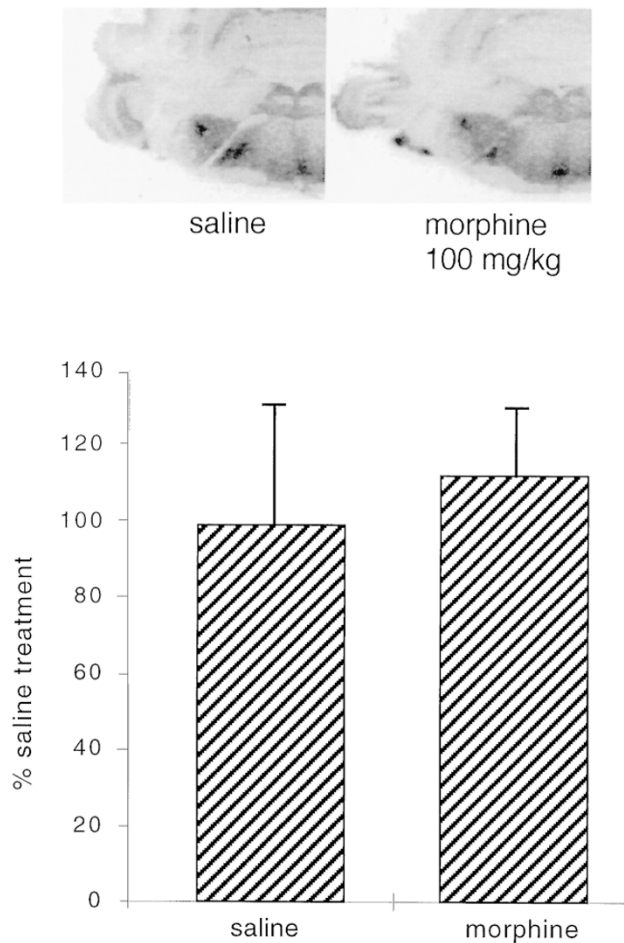


Figure 3. Changes in [125 I] galanin binding in the LC of mice 5 h after a single injection of 100 mg/kg morphine ($n = 5$). A single injection of a high dose of morphine resulted in no significant change in galanin binding in the LC. Area quantitated is shown in Figure 2.

dissected immediately after the animals were sacrificed and frozen at -80°C .

In Situ Hybridization. Four groups of mice were used for in situ hybridization studies. First, mice that received chronic saline and were sacrificed 3 h after a saline injection ($n = 10$). Second, mice that received chronic saline and were sacrificed 3 h after a 1 mg/kg naltrexone injection ($n = 6$). Third, mice that received chronic morphine as described above and were sacrificed 3 h after saline injection ($n = 6$). Fourth, mice that received chronic morphine and were sacrificed 3 h after 1 mg/kg naltrexone injection ($n = 11$).

Autoradiography Studies

Galanin autoradiography was adapted from previously published protocols (Ikeda et al. 1995). Briefly, 14-

micron brain sections were cut at the cryostat and collected on gelatinized slides at the level of the LC (Bregma -5.34 mm to -5.68 mm), ventral tegmental area (VTA) (Bregma -3.28 mm to -3.52), or nAc (Bregma $+1.34$ mm to $+1.70$ mm) (Franklin and Paxinos 1997). Sections were allowed to dry for 20 min at room temp and then were returned to the cryostat. Slides were stored at -80°C for no longer than 72 h. At the time of the experiment, slides were equilibrated to room temp. Two sections per slide were selected, and two slides from each treatment were used. Slides were preincubated in a humid chamber with 40 μl /section of buffer A (50 mm Tris pH 7.4, 5 mm MgCl_2 , 2 mm EGTA) for 30 min. After removal of preincubation solution, sections were incubated for 60 min at room temperature with buffer A plus 20 $\mu\text{g}/\text{ml}$ leupeptin, 1% BSA, and 0.25 nm [125 I]-galanin (Amersham, Piscataway, NJ). In a separate experiment, sections from either saline-treated mice or mice chronically treated with morphine and administered naltrexone were incubated with 0.01, 0.05, 0.1, 0.25 or 0.5 nm [125 I]-galanin to determine whether changes in binding were attributable to changes in B_{max} . Following incubation, slides were washed 2×1 min in ice cold 50 mm Tris pH 7.4 and dipped briefly in ice cold dH_2O . Slides were dried and exposed to Hyperfilm (Amersham) for 96–120 h. Nonspecific binding was determined in the presence of 250 nm cold galanin. Quantitation was performed by comparison to calibrated iodinated standards using image analysis (NIH Image) of autoradiograms according to established protocols (Benfenati et al. 1986). Briefly, areas to be quantitated were determined according to an adult mouse brain atlas (Franklin and Paxinos 1997), circled in NIH image, and an area of similar size was quantitated for each brain section. For each brain section, left and right sides were averaged. Four brain sections were quantitated per individual and averaged. For every section, white matter was used to determine background. Data are expressed as specific binding (total binding minus nonspecific binding), with specific binding in the saline-treated animals considered to be 100%.

In Situ Hybridization Studies

An 836 base pair fragment of exon 1 was amplified by PCR from a genomic clone encoding GalR1 (Zachariou and Picciotto 1999) and subcloned into pBluescript SK (Stratagene, La Jolla, CA). No homology was seen between this fragment and other galanin receptor sequences. PCR primers used were: TATAAGCTTAGG-GTCACCGGTCCTACTA (antisense) and AACTGTCAG-CTGGCGGTGGGCTTCAT (sense). Antisense riboprobes for GalR1 were transcribed using T7 DNA polymerase (Boehringer Mannheim, Indianapolis, IN) in the presence of [^{35}S]CTP (Dupont NEN, Boston MA). Residual

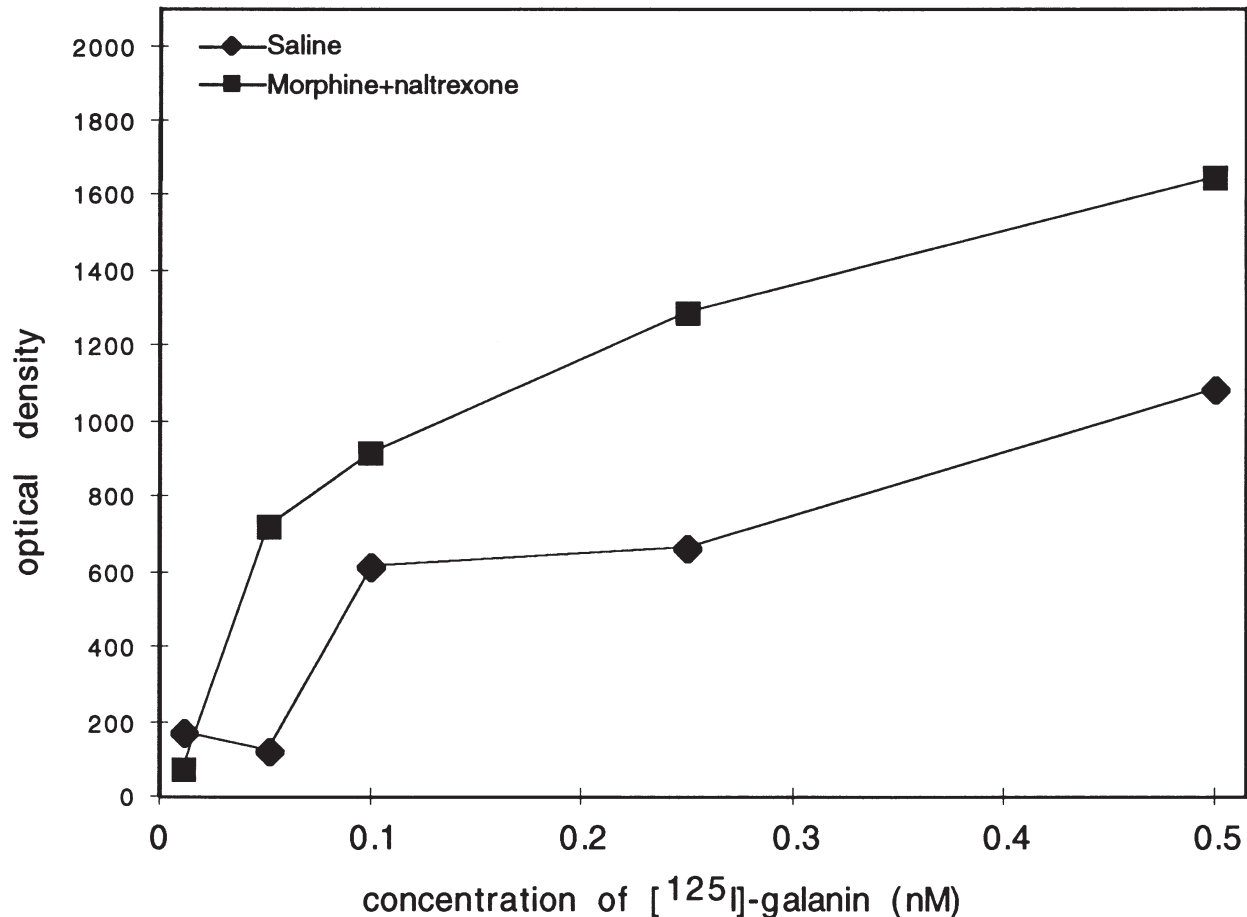


Figure 4. [¹²⁵I] Galanin binding in the LC at several concentrations following chronic morphine treatment with naltrexone precipitated withdrawal or chronic saline treatment ($n = 4$ for each group). Incubation with increasing doses of galanin resulted in saturation curves of binding in the LC that plateaued at a level approximately twofold higher following naltrexone-precipitated morphine withdrawal as compared to chronic saline treatment.

DNA was removed by digestion with DNase (Boehringer Mannheim) and riboprobes were purified on a Nucrap column (Stratagene).

Following drug treatments, animals were sacrificed by rapid decapitation, brains were removed and frozen in dry ice. 14- μ m sections were cut on a cryostat, mounted on Probe On slides (Fisher, Pittsburgh, PA), and stored at -80°C . Slides were fixed in paraformaldehyde (4%) immediately before hybridization. Slides were hybridized with the GalR1 sense or antisense riboprobes (6×10^7 (counts per min)cpm of labeled probe per 100 μ l) in humidified boxes overnight at 52°C . Sections were then rinsed in 2X SSC (concentrations), treated with RNase A (20 mg/ml, 30 min, 37°C), washed in 0.2X SSC for 10 min, 0.2X SSC (55°C) for 20 min and 0.1X SSC (55°C) for 15 min, then rinsed briefly in milli-Q water, and dried. Sections were exposed to b-max Hyperfilm (Amersham) for 3 weeks. Quantitation was performed by image analysis of autoradiograms exactly as described above for binding studies. No labeling was seen in the sense probe controls.

Statistical Analysis

Data for autoradiography and in situ hybridization experiments are presented as average \pm SEM. Statistical analysis was performed using Student's t -test or analysis of variance (ANOVA) for repeated measures followed by the least significant difference of the means multiple range post hoc test, with $p < .05$ considered to be a significant change.

RESULTS

Following chronic morphine treatment, naltrexone was injected, and withdrawal behavior was observed for 30 min ($n = 8$). Typical withdrawal signs were observed (weight loss, teeth chattering, ptosis, jumping) and as has been reported for mice (Maldonado et al. 1996), jumping was the most prominent symptom (Figure 1). Chronic morphine treatment in the absence of withdrawal and naltrexone treatment alone had no effect on

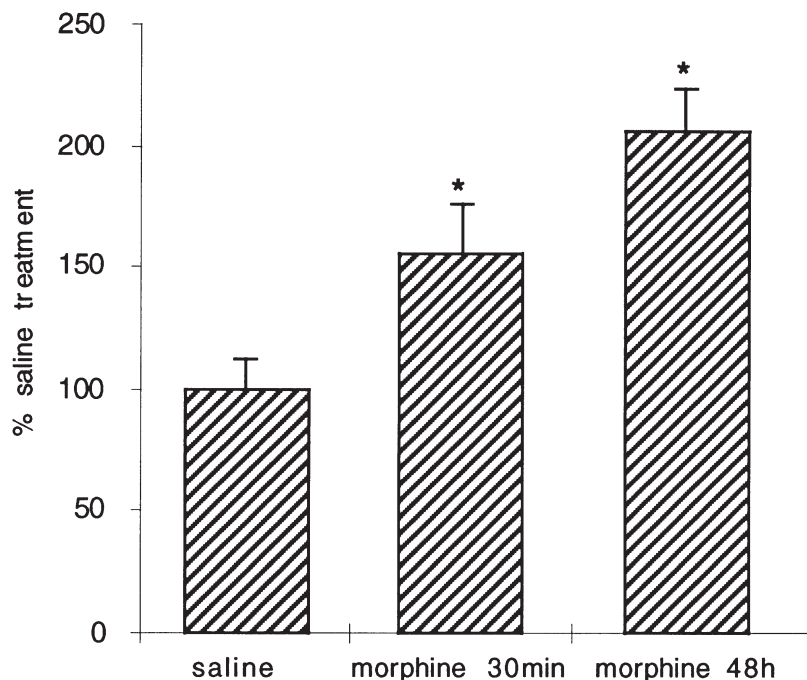


Figure 5. Changes in [I^{125}] galanin binding in the LC of mice that received 10 mg/kg morphine for 10 days and were sacrificed 30 min or 48 h after the last morphine injection ($n = 5$ for each group). Chronic intermittent morphine treatment resulted in persistent increases in galanin binding in the LC that were present 30 min following the last injection and were further increased 48 h after the last injection. * $p < .05$ as compared to saline controls.

galanin binding in the LC. Five h after naltrexone-precipitated withdrawal, galanin binding sites in the LC increased $155 \pm 59\%$ as compared to saline-injected controls ($p < .05$), and at 24 h, galanin binding sites were still elevated (61 ± 24 , $p < .05$) (Figure 2). Acute treatment with 100 mg/kg morphine SC failed to induce any changes in galanin binding in the LC 5 h following the injection (Figure 3).

Another set of animals was either treated with chronic morphine and sacrificed 5 h after naltrexone-precipitated withdrawal or given repeated saline injections (Figure 4). Brain sections at the level of the LC were then incubated with multiple concentrations of iodinated galanin to determine whether changes in galanin binding were attributable to an increase in B_{max} or an alteration in binding affinity. Binding seemed to saturate near 0.2 nm galanin, the K_d for binding to GalR1. At saturation, binding was approximately 2-fold lower in saline-treated animals.

In another set of animals, 10 mg/kg morphine was administered once a day for 10 days (Figure 5). Thirty min following the last morphine injection, galanin binding sites were upregulated in the LC ($55 \pm 20\%$ increase over vehicle treatment ($p < .05$), and 48 h following the last morphine injection, galanin binding sites were further increased (105 ± 17 , $p < .05$).

Levels of GalR1 mRNA were then examined in the LC following precipitated morphine withdrawal to determine whether upregulation of galanin binding sites

was attributable to increased GalR1 mRNA levels. A $120 \pm 47\%$ increase in GalR1 mRNA was seen 3 h after precipitated withdrawal (Figure 6). No significant changes in GalR1 mRNA levels were seen following morphine or naltrexone treatment alone.

Galanin binding sites were also examined following morphine treatment in nAc and VTA, two brain areas associated with the development of morphine reinforcement rather than physical withdrawal. The effects of saline, naltrexone alone, chronic morphine treatment, or naltrexone-precipitated withdrawal on galanin binding in the nAc are shown in Figure 7. Five h following naltrexone treatment alone, there was a $43 \pm 12\%$ increase ($p < .05$) in galanin receptor binding in the nAc. A similar increase ($28 \pm 12\%$) was seen in chronic morphine-treated animals 5 h following acute naltrexone treatment, suggesting that this change was attributable to naltrexone treatment rather than precipitated withdrawal. Twenty-four h following naltrexone treatment, galanin receptor levels had returned to baseline values, implying that regulation of galanin receptor levels by opioids is dynamic. There was no change in galanin binding in the nAc following chronic morphine treatment in the absence of naltrexone administration. In addition, naltrexone, chronic morphine or naltrexone-precipitated withdrawal had no effect on galanin binding in the VTA (Figure 8), nor were any changes in galanin binding sites observed in the VTA or nAc following repeated administration of 10 mg/kg morphine (data not

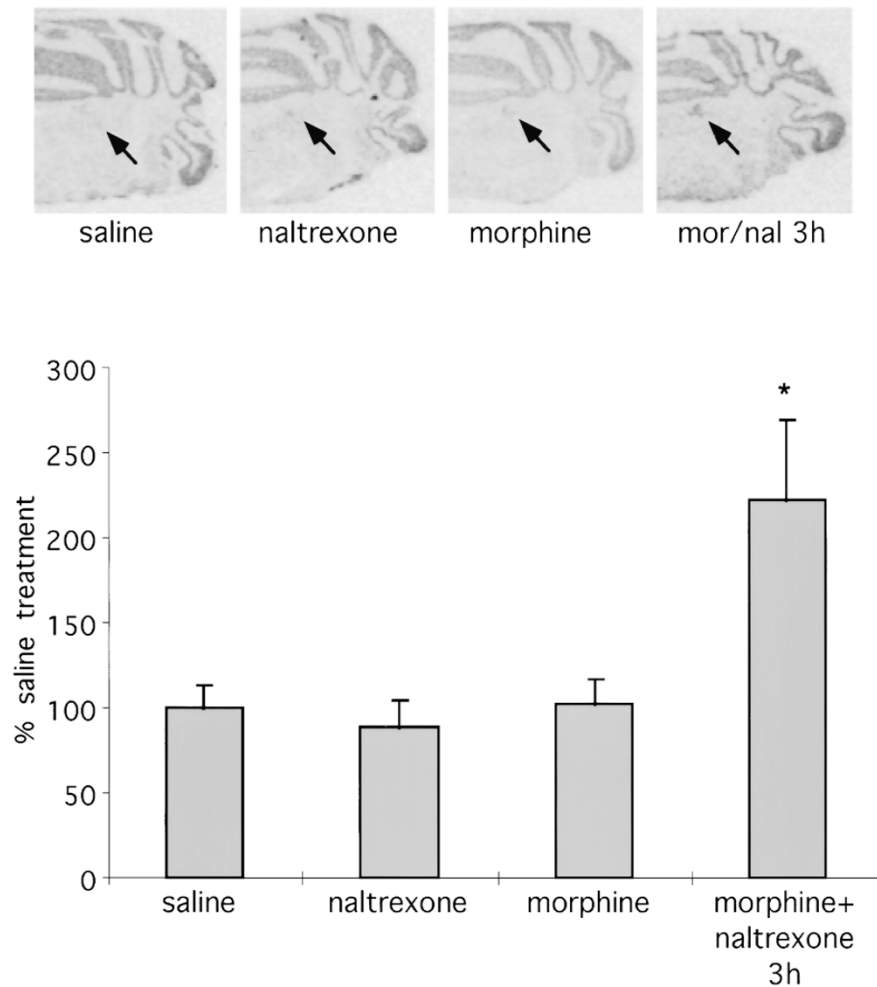


Figure 6. Changes in GalR1 mRNA in the LC. In situ hybridization was performed following chronic saline with a challenge saline injection ($n = 10$), following chronic saline with a challenge naltrexone injection ($n = 6$), following chronic morphine treatment with a challenge saline injection ($n = 6$), or following chronic morphine treatment with a challenge naltrexone injection ($n = 11$). A significant increase in GalR1 mRNA levels was seen only following naltrexone-precipitated morphine withdrawal. LC is indicated with the large arrow. $*p < .05$ as compared to saline group.

shown). In situ hybridization studies showed that there was no detectable GalR1 mRNA in the VTA, suggesting that galanin binding in this brain area is either due to GalR1 protein transported to the VTA from another brain area or to another galanin receptor subtype. GalR1 mRNA is moderately expressed in the striatum, and is expressed in lower amounts in the nucleus accumbens in a pattern resembling that of cholinergic interneurons (Figure 9). The sparse distribution of GalR1 mRNA in these brain areas precluded accurate quantitation following morphine treatment.

DISCUSSION

Using receptor autoradiography, we have shown that opiate withdrawal results in upregulation of galanin binding sites in the LC. In addition, mRNA encoding

one subtype of galanin receptor, GalR1, was upregulated in the LC 3 h following withdrawal, either because of increased transcription or mRNA stabilization. These data suggest that opioid withdrawal regulates GalR1 mRNA levels and that the changes in galanin binding seen in this brain region may reflect changes in galanin receptor gene expression. The results of the current study on galanin receptor levels are in contrast to a previous finding that galanin peptide mRNA levels do not change following morphine withdrawal (Holmes et al. 1995b). One possibility for this discrepancy between peptide and receptor regulation is that galanin peptide levels could be regulated post-transcriptionally during opiate withdrawal. It is also possible that galanin saturates available receptors under these conditions, so that galanin-mediated activity can be regulated at the level of its receptor. Both reserpine treatment (Kadowaki and Emson 1992) and chronic stress (Holmes et al. 1995a)

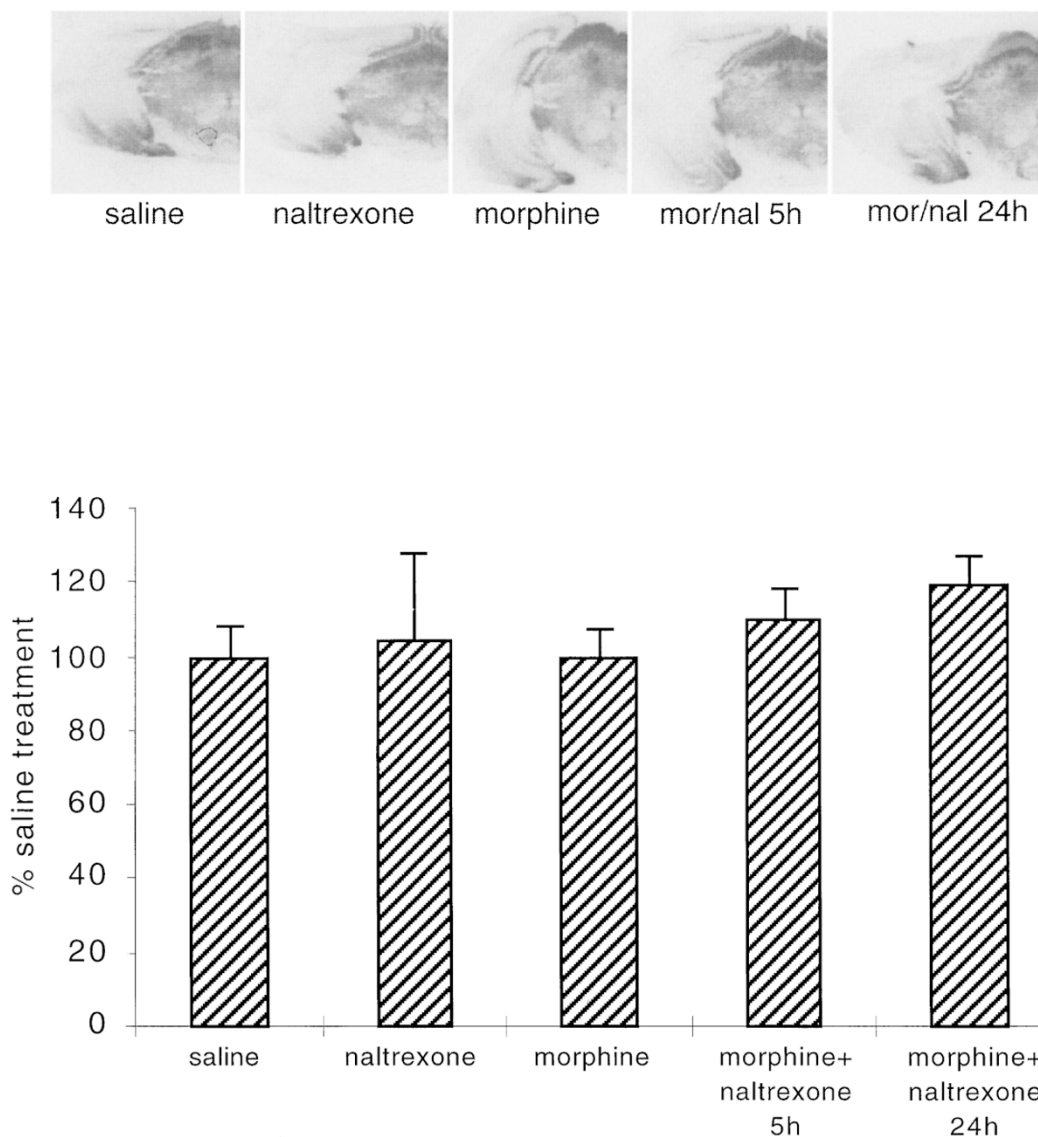


Figure 7. Changes in [125 I] galanin binding in the VTA following saline ($n = 5$), naltrexone ($n = 5$), chronic morphine ($n = 4$), and two time points (5 and 24 h) following naltrexone precipitated withdrawal ($n = 5$ for each group). No change was seen in galanin binding in the VTA following any of the treatments. Area indicated with a dashed line was quantitated.

can increase galanin peptide mRNA levels in the LC, however, indicating that either severe perturbations of the LC, or chronic increases in LC firing rate are able to regulate galanin mRNA levels. It is, therefore, possible that the 3-h time point regarded was too short following induction of withdrawal to observe changes, or that greater perturbations are required to regulate peptide than receptor levels.

GalR1 mRNA is expressed at low levels but above background in the LC under basal conditions in our experiments. Even following a dramatic increase during withdrawal, GalR1 mRNA levels were low compared to tyrosine hydroxylase mRNA (data not shown). Other investigators have also reported GalR1 mRNA expres-

sion in the LC (Gustafson et al. 1996), although a second study found no GalR1 mRNA in the LC but rather saw GalR1 expression in the neighboring Barrington's nucleus (Xu et al. 1998). In the negative study, oligonucleotide probes were used to detect GalR1 message. Oligonucleotides can be less sensitive than riboprobes, which were used in the present study, perhaps accounting for the discrepancy.

A number of studies have shown that acute morphine treatment decreases the activity of G-proteins and adenylate cyclase and inhibits cAMP-dependent protein phosphorylation in brain regions associated with drug reinforcement and dependence (Duman et al. 1988; Guitart and Nestler 1989). Chronic morphine

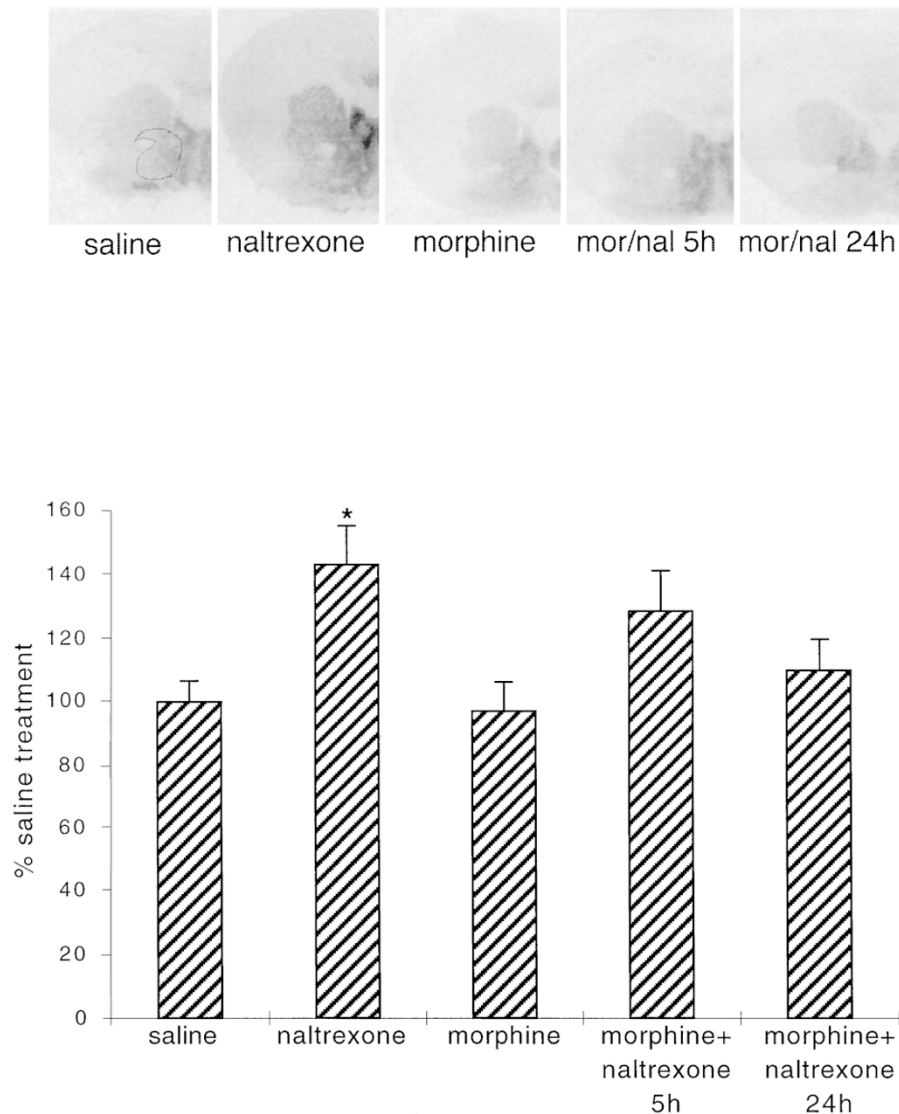


Figure 8. Changes in [125 I] galanin binding in the nAc following saline ($n = 10$), naltrexone ($n = 10$), chronic morphine ($n = 10$), and two time points (5 and 24 h) following naltrexone precipitated withdrawal ($n = 10$ for each group). Galanin binding in the nAc was significantly increased following naltrexone treatment alone but not with any other treatment. Area circled with a dashed line was quantitated. $*p < .05$ as compared to saline controls.

treatment results in compensatory upregulation of these systems in the nAc and LC (Nestler 1992), resulting in increased firing of LC neurons, opiate dependence, and the expression of withdrawal symptoms (Nestler et al. 1993; Rasmussen et al. 1990). In the current study, we show that galanin receptor levels do not change significantly during chronic morphine treatment alone. Previous studies have shown that opioid receptor levels are also unchanged by this treatment (Loh and Smith 1990). In contrast, 5 h following naltrexone-precipitated withdrawal, galanin receptor binding increased by approximately 150%, and GalR1 mRNA levels increased approximately 120%. The increase in galanin binding and GalR1 mRNA levels could be interpreted as a compensatory mechanism to decrease fir-

ing of LC neurons and could be related to the changes in the cAMP system seen following withdrawal.

Alternatively, it is possible that galanin peptide levels decrease during withdrawal in the absence of a change in mRNA levels, leading to upregulation of galanin receptors. This possibility could be examined in the future by immunostaining for the galanin peptide.

Both galanin binding sites and μ -opioid receptors are present in the VTA, but neither morphine nor naltrexone administration alone affected galanin receptor levels in this area. Similarly, other studies have shown that, whereas, G-protein and adenylate cyclase levels in the nAc can be altered by morphine treatment, no change in components of the cAMP pathway is seen in the VTA following chronic morphine treatment (Nes-

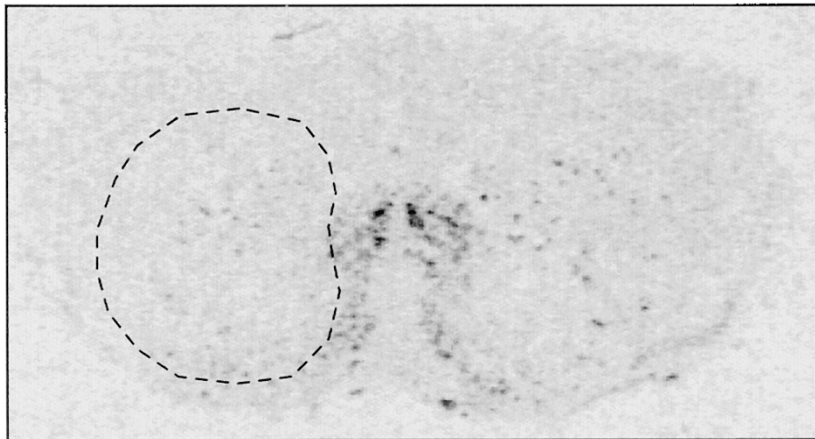


Figure 9. Distribution of mRNA encoding GalR1 in the striatum. In situ hybridization with a riboprobe specific to GalR1 was used to detect mRNA encoding this receptor in the striatum and nAc. The punctate labeling is reminiscent of the distribution of cholinergic interneurons in this brain area. Striatum and nAc are indicated by dotted lines.

tlar et al. 1993). Galanin receptor levels may, therefore, be responsive to alterations in the cAMP pathway, or may be subject to similar types of regulation as the components of that pathway.

Chronic morphine treatment in the absence of withdrawal failed to increase levels of galanin binding significantly, although repeated intermittent morphine administration increased galanin binding sites. Although these animals showed no physical signs of withdrawal, it is likely that tolerance to morphine had developed, and, therefore, that the animals were morphine-dependent (Etemadzadeh 1994; Marek et al. 1991). This could imply that regulation of the galanin receptor reflects the development of morphine tolerance and dependence. A single large dose of morphine (100 mg/kg) had no effect on galanin binding, suggesting that repeated morphine administration is required for the upregulation of the galanin receptor in the LC. The neurochemical changes in the LC underlying tolerance may, therefore, be involved in the alteration in galanin binding sites, but the exact mechanism of this phenomenon remains to be investigated.

Administration of naltrexone resulted in an increase in galanin binding sites in the nAc, suggesting that galanin receptors in this area are under tonic opioidergic regulation. This mechanism of regulation seems to be distinct from that seen in other brain areas, because no change in galanin binding levels was seen following naltrexone administration in either LC or VTA. Previous studies have shown that icv galanin can attenuate morphine place preference (Zachariou et al. 1999). These data taken together with the binding data suggest that interaction between the opiate system and the galaninergic system at the level of the nAc is likely to be related to opiate reinforcement rather than withdrawal.

In summary, this study has shown that galanin binding sites and GalR1 mRNA levels are upregulated in the LC following morphine withdrawal. Although the mechanism of this upregulation is not yet known, it is possible that changes in the cAMP pathway that occur

following drug dependence and withdrawal may be involved. Future studies will be necessary to characterize whether cAMP pathways are involved in this regulation.

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