EJN fens EUROPEAN JOURNAL OF NEUROSCIENCE

European Journal of Neuroscience, Vol. 37, pp. 1541–1549, 2013 doi:10.1111/ejn.12151

BEHAVIORAL NEUROSCIENCE

Galanin-induced decreases in nucleus accumbens/striatum excitatory postsynaptic potentials and morphine conditioned place preference require both galanin receptor 1 and galanin receptor 2

Emily B. Einstein,[1,2] Yukiko Asaka,[1] Mark F. Yeckel,[3] Michael J. Higley[3,4] and Marina R. Picciotto[1,3,4]
[1]Division of Molecular Psychiatry, Department of Psychiatry, Yale University School of Medicine, 34 Park Street – 3rd floor research, New Haven, CT, 06508, USA
[2]Interdepartmental Neuroscience Program, Yale University School of Medicine, New Haven, CT, USA
[3]Department of Neurobiology, Yale University School of Medicine, New Haven, CT, USA
[4]Program in Cellular Neuroscience, Neurodegeneration and Repair, Yale University School of Medicine, New Haven, CT, USA

Keywords: addiction, extracellular recording, mice, morphine, patch clamp

Abstract

The neuropeptide galanin has been shown to alter the rewarding properties of morphine. To identify potential cellular mechanisms that might be involved in the ability of galanin to modulate opiate reward, we measured excitatory postsynaptic potentials (EPSPs), using both field and whole-cell recordings from striatal brain slices extracted from wild-type mice and mice lacking specific galanin receptor (GalR) subtypes. We found that galanin decreased the amplitude of EPSPs in both the dorsal striatum and nucleus accumbens. We then performed recordings in slices from knockout mice lacking either the GalR1 or GalR2 gene, and found that the ability of galanin to decrease EPSP amplitude was absent from both mouse lines, suggesting that both receptor subtypes are required for this effect. In order to determine whether behavioral responses to opiates were dependent on the same receptor subtypes, we tested GalR1 and GalR2 knockout mice for morphine conditioned place preference (CPP). Morphine CPP was significantly attenuated in both GalR1 and GalR2 knockout mice. These data suggest that mesolimbic excitatory signaling is significantly modulated by galanin in a GalR1-dependent and GalR2-dependent manner, and that morphine CPP is dependent on the same receptor subtypes.

Introduction

Opioids are extremely effective analgesics, and are the most widely prescribed class of drugs in the USA (Kuehn, 2007). Unfortunately, the actions of these drugs on the brain reward circuitry (Matthews & German, 1984; Shippenberg & Elmer, 1998) confer a substantial risk for abuse (Compton & Volkow, 2006). It is therefore critical to understand the endogenous mechanisms that modulate morphine reward, in order to identify novel pathways to combat the development of opiate addiction.

The neuropeptide galanin interacts with morphine in several behavioral paradigms. Galanin and morphine can both alleviate pain (Wiesenfeld-Hallin *et al.*, 1990; Hobson *et al.*, 2006; Hulse *et al.*, 2011), but, in combination, they produce significantly greater antinociception than can be explained by an additive effect (Wiesenfeld-Hallin *et al.*, 1990; Przewłocka *et al.*, 1995; Zhang *et al.*, 2000; Hua *et al.*, 2004; Sun & Yu, 2005). In contrast, galanin can interfere with the rewarding properties of morphine. For example, in a conditioned place preference (CPP) paradigm, intracerebroventricular infusion of galanin decreased CPP to a threshold dose (Zachariou *et al.*, 1999). Similarly, mice lacking the galanin peptide showed morphine CPP at a lower dose than their wild-type littermates (Hawes *et al.*, 2008), indicating a potential role for galanin in morphine reward processing.

Morphine CPP requires activity of the mesocorticolimbic system (Koob, 1992; Carlezon & Wise, 1996). Both the dorsal striatum (DS) and the nucleus accumbens (NAc) are critical for the integration of dopaminergic signaling of the midbrain with glutamatergic inputs from the prefrontal cortex, hippocampus, and amygdala (Koob, 1992; Chao & Nestler, 2004; Carlezon & Thomas, 2009; Stuber *et al.*, 2012). The medium spiny neuron (MSN) outputs from these areas influence drug taking and habit formation (Koob & Volkow, 2010). Galanin signals through three receptor subtypes, galanin receptor (GalR)1, GalR2, and GalR3, all of which can couple to G_i signaling cascades (Smith *et al.*, 1997, 1998; Branchek *et al.*, 2000; Lang *et al.*, 2007). So far, the inhibitory effects of galanin have

Correspondence: Dr R. Picciotto, [1]Division of Molecular Psychiatry, as above.
E-mail: marina.picciotto@yale.edu

Received 4 October 2012, revised 3 January 2013, accepted 9 January 2013

been characterised in the dorsal raphe, locus coeruleus, hypothalamus, and hippocampus (Xu *et al.*, 2005; Picciotto *et al.*, 2010). Despite reports that galanin can alter dopamine dynamics (Tsuda *et al.*, 1998; Ericson & Ahlenius, 1999), a direct effect of galanin on the excitability of neurons in the mesocorticolimbic system has never been shown.

In order to identify physiological mechanisms that could underlie the interplay between galanin signaling and morphine reward, we recorded excitatory postsynaptic field potentials (fEPSPs) in the DS and in the NAc before and after bath application of galanin. We also recorded the effect of galanin on excitatory postsynaptic potentials (EPSPs) from individual MSNs in GalR1 and GalR2 knockout (KO) mice and their wild-type (WT) siblings. Finally, to determine whether the same GalR subtypes were involved in the effects of galanin on mesolimbic physiology and morphine reward, we tested GalR1 or GalR2 KO mice and their WT siblings in a morphine CPP paradigm. These experiments revealed a novel interaction between the morphine and galanin systems that provides a mechanistic basis for the role of individual GalR subtypes in modulation of the mesolimbic system.

Materials and methods

Animals

All mice were housed under standard laboratory conditions (21 ± 2 °C, lights on from 07:00 h to 19:00 h) in Sealsafe Plus individually ventilated cages (Tecniplast, Buguggiate, Italy), and given *ad libitum* access to chow (Harlan Teklad #2018) and water. For electrophysiological recordings in WT mice, C57BL/6J litters were obtained from Jackson Laboratories (Bar Harbor, ME, USA), and housed together with the dam until weaning at postnatal day (P) 28. Mice with an inactivating mutation in exon 1 of the GalR1 gene were generated as described previously (Tsuda *et al.*, 1998; Ericson & Ahlenius, 1999), and backcrossed onto a C57Bl/6J background for at least 10 generations. Mice with an early termination mutation in the intron of the GalR2 gene were generated as described previously (Hobson *et al.*, 2006) and backcrossed onto a C57Bl/6J background for at least 10 generations. Heterozygous mating pairs of $GalR1^{+/-}$ or $GalR2^{+/-}$ mice were used to generate WT GalR1 and GalR1 KO or WT GalR2 and GalR2 KO littermates, respectively, for behavioral testing. In some physiology experiments, GalR1 or GalR2 KO mice were generated from homozygous breeding pairs. Adult mice were housed together (two to five per cage), and habituated to the colony for at least 1 week before behavioral testing. Both male and female mice were tested in behavioral studies. Male mice were used for field recording studies in WT mice. In patch clamp studies, recordings were performed in slices from male and female WT, GalR1 KO and GalR2 KO mice. There was no significant difference between males and females in these recordings, so the data from both sexes were combined for statistical analysis. All animal procedures were approved by the Yale Animal Care and Use Committee, and conducted in compliance with the guidelines laid down by the National Institutes of Health regarding the care and use of animals for experimental procedures.

Drugs

Morphine-HCl (NIDA drug supply program) was dissolved in 0.9% saline, and delivered subcutaneously at 0.01 mL/g. Galanin (1–29; rat–mouse; Tocris, Bristol, UK) and galnon (Tocris) were dissolved in artificial cerebrospinal fluid (ACSF) to a stock concentration of 1 mM. Picrotoxin (Sigma-Aldrich, St Louis, MO, USA) and CGP55845 (Tocris) were dissolved in dimethylsulfoxide to respective stock concentrations of 100 mM and 50 mM.

Slice preparation

Acute striatal slices were prepared from P19 to P33 mice. Mice were anesthetised with isoflurane and decapitated, and the brain was rapidly dissected into ice-cold cutting solution containing 200 mM sucrose, 2.5 mM KCl, 1.3 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$ and 11 mM dextrose for field recordings, or 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 25 mM glucose, 2.5 mM KCl, 7.0 mM $MgCl_2$, 110 mM choline-Cl, 11.6 mM ascorbate, 3.1 mM pyruvate and 0.5 mM $CaCl_2$ for patch clamp recordings. Coronal or parasagittal slices were cut to a thickness of 300 μm with a Leica VT1000S Vibratome, and then transferred to oxygenated ACSF containing 127 mM NaCl, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 25 mM glucose, 2.5 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$. Slices were incubated at 37 °C for 30 min before being allowed to recover at room temperature for an additional 30 min.

For recordings, slices were submerged in a recording chamber and continuously superfused with room temperature ACSF, which was constantly bubbled with 95% O_2/5% CO_2. Responses were collected for a total duration of 40–50 min for fEPSPs and for 20–30 min for EPSPs. Recording ACSF contained 100 μM picrotoxin to block $GABA_A$ receptors and 2 μM CGP55845 to block $GABA_B$ receptors. Galanin was bath applied at a concentration of 100 nM or 1 μM, and galnon was bath applied at a concentration of 1 μM.

Field potential recordings

fEPSP recordings were performed in the striatum to measure responses evoked by stimulation of excitatory cortical afferents in the corpus callosum (for experiments in the DS) or in the NAc (for experiments in the NAc), with bipolar tungsten electrodes (FHC, Bowdoinham, ME, USA). Test stimuli were applied at a low frequency to elicit an fEPSP amplitude of 30–50% of maximum. Synaptic responses were recorded extracellularly with glass microelectrodes (0.5–1 MΩ) filled with 2 M NaCl, placed 200–600 μm ventral to the stimulation point. Signals were amplified with a DP-301 differential amplifier (Warner Instrument Corporation, Hamden, CT, USA) and digitised at 10 kHz for analysis with PCLAMP 9.0 software (Axon Instruments, Union City, CA, USA). fEPSPs showed a wave shape characteristic of cortico-striatal activation. These waves consist of two negative potentials, N1 and N2, which have been associated with direct and synaptic activation, respectively (Lovinger *et al.*, 1993; Sergeeva *et al.*, 2003). fEPSPs were quantified with the initial slope of N2 as calculated between points at approximately 10 and 30% of the negative potential, in order to isolate initial synaptic input. To ensure that the effects of galanin were not attributable to run-down, we also tested the effects of the small-molecule GalR agonist galnon (Saar *et al.*, 2002). To control for wash-in of galanin, statistical analyses were performed on the last five evoked responses of the baseline and galanin epochs for each experiment. All data were analysed with repeated measures ANOVA. No significant effect of, or interaction with, time was detected, so responses were averaged for two-tailed, paired sample *t*-tests, and the level of significance was set at $P < 0.05$.

Whole-cell current clamp recordings

Single-cell EPSPs were evoked by stimulation of excitatory cortical afferents with theta glass stimulating electrodes. Stimuli were applied to evoke responses between 5 and 10 mV. Whole-cell

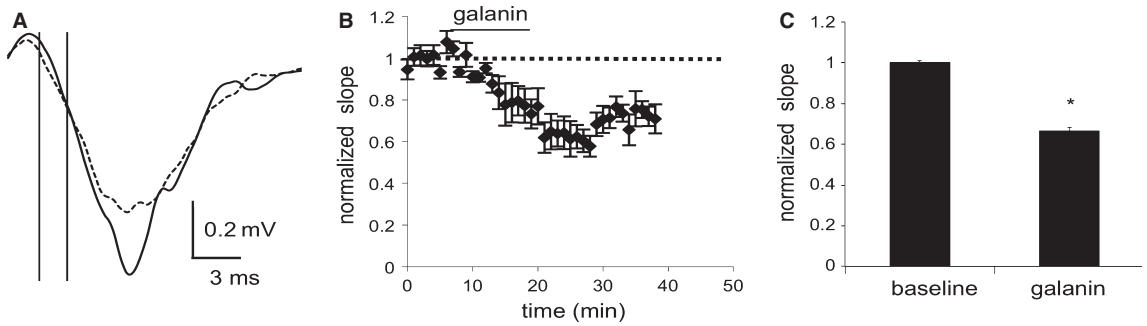

FIG. 1. Field recordings in the DS of C57BL/6J mice show a decrease in fEPSPs after application of galanin (100 nM). (A) The average of four consecutive traces during the baseline (solid line) and galanin (dashed line) epochs of a representative experiment. The initial slope was calculated from the points at which the vertical lines intersect with the trace. (B) The combined normalised amplitudes (n = 6 slices from six male mice) before and after galanin application. (C) The mean of the averaged last five responses of the baseline and galanin epochs. Data are expressed as mean ± standard error (SE) of the mean. *P < 0.05.

recordings were obtained from MSNs identified with video-IR/DIC. Glass recording electrodes (2–4 MΩ) were filled with internal solution containing 135 mM $KMeSO_3$, 10 mM Hepes, 4 mM $MgCl_2$, 4 mM Na_2ATP, 0.4 mM NaGTP, and 10 mM disodium creatine phosphate, adjusted to pH 7.4 with KOH. Data were collected with a Molecular Devices Multiclamp 700B, and digitised at 10 kHz. To control for wash-in of galanin, statistical analyses were performed on the maximum amplitude of the last five evoked responses of the baseline and galanin epochs for each experiment. All data were analysed with repeated measures ANOVA. No significant effect of, or interaction with, time was detected, so responses were averaged for two-tailed, paired sample t-tests, and the level of significance was set at P < 0.05.

CPP

Mice (aged 3–5 months) were habituated to handling once a day for 3 days prior to testing. The CPP paradigm was carried out exactly as has been described previously (Narasimhaiah *et al.*, 2009; Neugebauer *et al.*, 2011), by use of modified three-chamber boxes (ENV-256C; Medical Associates, St Albans, VT, USA) with a central gray chamber and two black conditioning chambers distinguishable by their respective grid and bar floors. Movement within and among chambers was quantified by photocell beam breaks, and time spent in each chamber was recorded with MED-PC IV software. Day 1 of of testing consisted of a midday pre-test (beginning at approximately 11:00 h), during which mice were placed in the central chamber and allowed to freely explore all chambers for 15 min. Mice that spent > 70% of the test period in one chamber were excluded from the experiment (n = 3 of 142). On days 2–4, mice received conditioning sessions. During morning sessions (beginning at approximately 09:00 h), mice were given a subcutaneous injection of 0.9% saline (0.01 mL/g) immediately before confinement in the saline-paired chamber for 30 min. For afternoon sessions (beginning at approximately 13:00 h), mice were given a subcutaneous injection of drug (0, 3 or 5 mg/kg morphine) immediately before confinement for 30 min in the drug-paired chamber. Mice within the treatment and genotype groups were counterbalanced for bar/grid floor drug chamber pairing. On day 5, a post-test was carried out at a time intermediate between the conditioning sessions, to avoid any associations with time of saline or drug administration, in which the mice were once again placed in the central chamber and allowed to freely explore for 30 min. Data are expressed as a difference score, which has been calculated by subtracting the post-test time spent in the saline-paired chamber from the post-test time spent in the drug-paired chamber. This difference score was then corrected by the average of the difference score from saline-treated mice. Because of the large number of mice in the behavioral studies, multiple cohorts were bred and tested at 3–5 months of age. Each cohort included all genotypes and morphine doses, and, to control for variability in preference ratios over time, each cohort was normalised to its own control group to allow pooling of the data. Data from GalR1 and GalR2 experiments were analysed with two-way ANOVA, with genotype and treatment as factors.

Results

Field potential recordings

To determine the effect of galanin on excitatory signaling in the striatum, we recorded fEPSPs in both the DS and the NAc. The initial fEPSP slope was significantly decreased by bath application of galanin (100 nM) in the DS. Repeated measures ANOVA did not reveal a significant effect of time or a significant interaction of time and treatment ($F_{4,32}$ = 1.626, P = 0.1917, and $F_{4,32}$ = 0.2684, P = 0.8961), but there was a significant effect of galanin treatment ($F_{1,8}$ = 7.255, P = 0.0273), which reduced the slope to 66% of baseline (n = 5 slices from five male mice; t_4 = 6.88, P = 0.0011; Fig. 1). In the NAc shell, repeated measures ANOVA again did not reveal a significant effect of time or a significant interaction of time and treatment ($F_{4,40}$ = 0.4151, P = 0.7967, and $F_{4,40}$ = 0.5301, P = 0.7143), but did show a significant effect of galanin treatment ($F_{1,10}$ = 32.88, P = 0.0002), which reduced the slope to 55% of baseline (n = 6 slices from six male mice; t_5 = 2.62, P = 0.0234; Fig. 2).

The ability of galnon to reduce the slope of the fEPSP in the DS and NAc, as well as the wash-out of this effect, suggests that the ability of galanin to decrease the fEPSP is unlikely to be attributable to run-down in the slice. For the DS, time and interaction of time and treatment were not significant ($F_{4,40}$ = 1.194, P = 0.0960, and $F_{4,40}$ = 2.121, P = 0.3284; Fig. 3), but there was a main effect of treatment ($F_{10,40}$ = 49.49, P < 0.0001; Fig. 3). The slope was reduced to 48% of baseline. For the NAc, time and interaction of time and treatment were also not significant ($F_{4,40}$ = 2.169, P = 0.0900, and $F_{4,40}$ = 1.358, P = 0.2656; Fig. 4), but there was again a main effect of treatment ($F_{10,40}$ = 22.51, P < 0.0001; Fig. 4). The slope was reduced to 67% of baseline.

Whole-cell current clamp recordings

To determine whether the inhibitory actions of galanin identified in field potential recordings were mediated by changes in synaptic inputs to MSNs, the amplitudes of electrically evoked EPSPs were compared before and after bath application of galanin. Initial studies

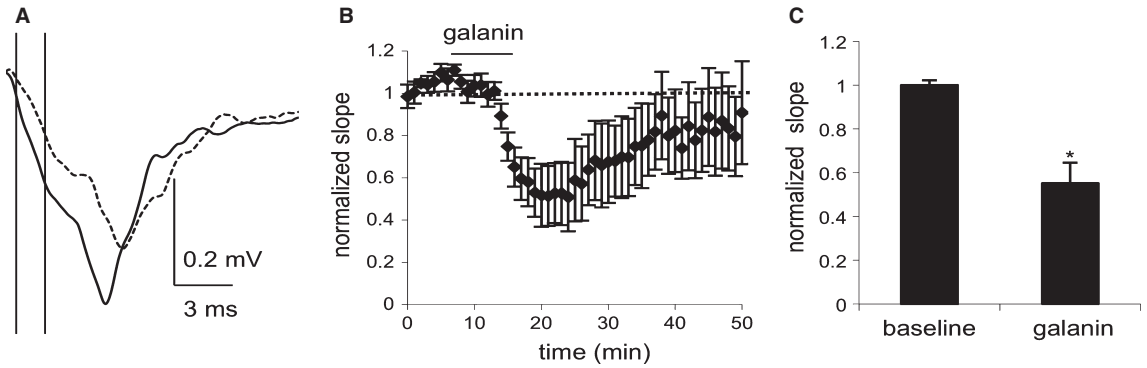

FIG. 2. Field recordings in the NAc of C57BL/6J mice show a decrease in fEPSPs after application of galanin (100 nM). (A) The average of four consecutive traces during the baseline (solid line) and galanin (dashed line) epochs of a representative experiment. The initial slope was calculated from the points at which the vertical lines intersect with the trace. (B) The combined normalised amplitudes (n = 6 slices from six male mice) before and after galanin application. (C)The mean of the averaged last five responses of the baseline and galanin epochs. Data are expressed as mean ± standard error (SE) of the mean. *P < 0.05.

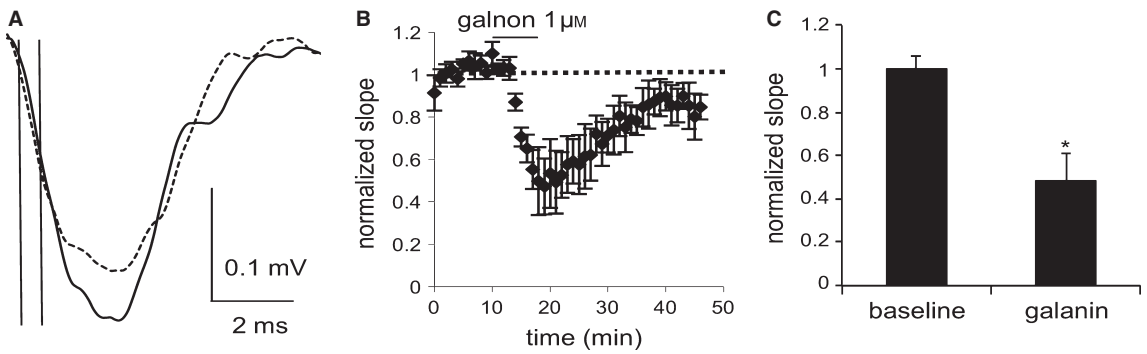

FIG. 3. Field recordings in the DS of C57BL/6J mice show a decrease in fEPSPs after application of galnon (1 μM). (A) The average of four consecutive traces during the baseline (solid line) and galnon (dashed line) epochs of a representative experiment. The initial slope was calculated from the points at which the vertical lines intersect with the trace. (B) The combined normalised amplitudes (n = 6 slices from six male mice) before and after galnon application. (C) The mean of the averaged last five responses of the baseline and galnon epochs. Data are expressed as mean ± standard error (SE) of the mean. *P < 0.05.

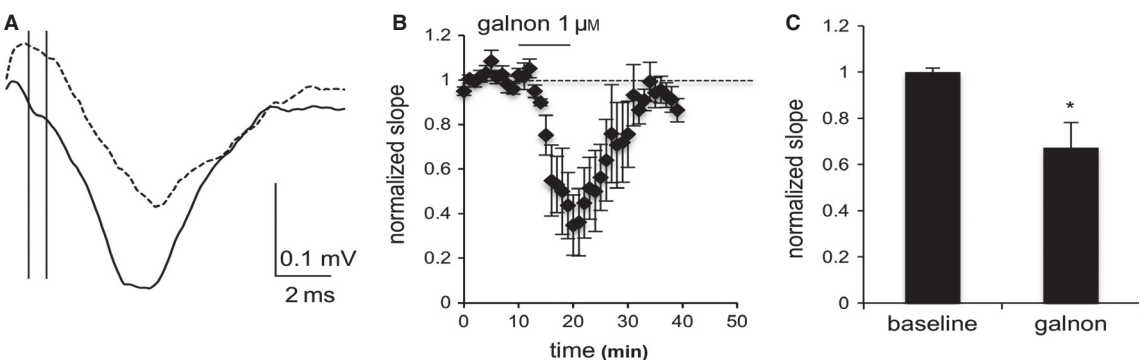

FIG. 4. Field recordings in the NAc of C57BL/6J mice show a decrease in fEPSPs after application of galnon (1 μM). (A) The average of four consecutive traces during the baseline (solid line) and galnon (dashed line) epochs of a representative experiment. The initial slope was calculated from the points at which the vertical lines intersect with the trace. (B) The combined normalised amplitudes (n = 6 slices from six male mice) before and after galnon application. (C) The mean of the averaged last five responses of the baseline and galnon epochs. Data are expressed as mean ± standard error (SE) of the mean. *P < 0.05.

with 100 nM galanin did not reveal consistent responses (not shown), so subsequent experiments were performed with 1 μM galanin. In MSNs of the DS, a significant decrease in EPSP amplitude was seen after galanin application. Repeated measures ANOVA did not reveal a significant effect of time or a significant interaction of time and treatment ($F_{4,48}$ = 1.951, P = 0.1171, and $F_{4,48}$ = 0.6389, P = 0.6373), but there was a significant effect of galanin treatment ($F_{1,12}$ = 92.48, P < 0.0001), which reduced the slope to 71% of baseline (n = 7 cells from six male mice; t_6 = 8.625, P = 0.0001; Fig. 5). Recordings from MSNs in the NAc also revealed a significant decrease in EPSP amplitude after galanin application. In NAc MSNs, repeated measures ANOVA again did not reveal a significant effect of time or a significant interaction of time and treatment ($F_{4,64}$ = 0.7856, P = 0.5388, and $F_{4,64}$ = 0.9662, P = 0.4322), but did show a significant effect of galanin treatment ($F_{1,16}$ = 39.35, P < 0.0001), which reduced the slope to 72% of baseline (n = 9

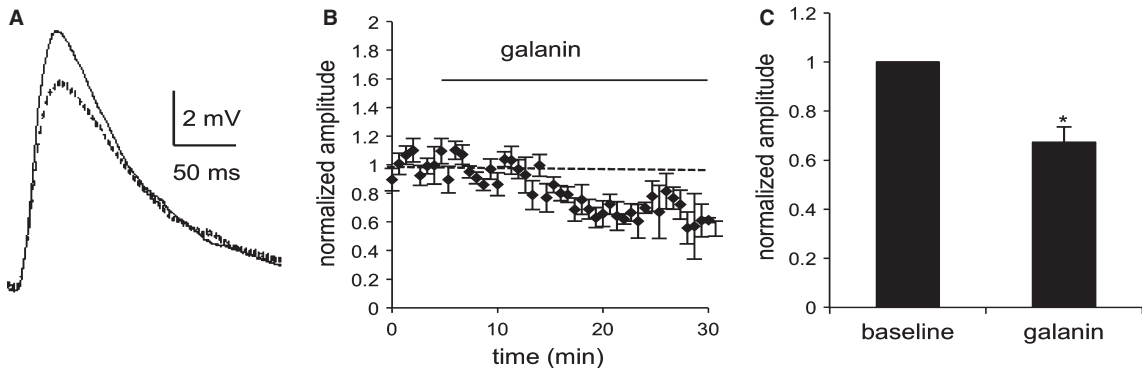

FIG. 5. MSNs in the DS of C57BL/6J mice show a decrease in EPSP amplitude after application of galanin (1 μM). (A) The average of five consecutive traces during the baseline (solid line) and galanin (dashed line) epochs of a representative experiment. (B) The combined normalised amplitudes (n = 7 cells from six male mice) before and after galanin (1 μM) application at t = 0. (C) The mean of the averaged last five responses of the baseline and galanin epochs. Data are expressed as mean ± standard error (SE) of the mean. *P < 0.05.

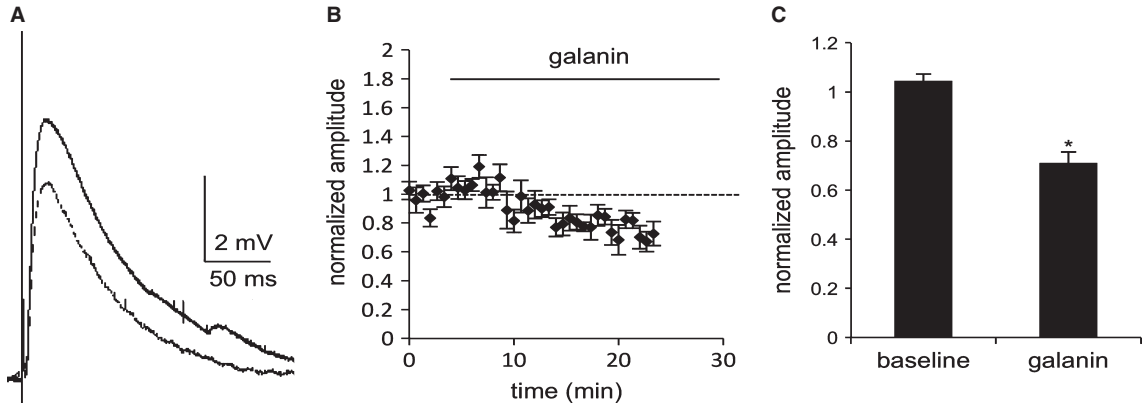

FIG. 6. MSNs in the NAc of C57BL/6J mice show a decrease in EPSP amplitude after application of galanin (1 μM). (A) The average of five consecutive traces during the baseline (solid line) and galanin (dashed line) epochs of a representative experiment. (B) The combined normalised amplitudes (n = 9 cells from seven male mice and two female mice) before and after galanin (1 μM) application at t = 0. (C) The mean of the averaged last five responses of the baseline and galanin epochs. Data are expressed as mean ± standard error (SE) of the mean. *P < 0.05.

cells from six male mice and three female mice; t_8 = 5.1410, P = 0.0009; Fig. 6). No significant changes in input resistance or membrane potential were observed in the DS or NAc. In the DS, the membrane potential was −89.7 ± 1.9 mV and the input resistance was 91 ± 8.3 MΩ, and in the NAc the membrane potential was −84.1 ± 2.4 mV and the input resistance was 130 ± 16.0 MΩ. The paired-pulse ratio was also assessed in both brain regions, and no significant difference was observed after application of galanin.

In order to identify the GalR subtypes involved in the ability of galanin to decrease the amplitude of the EPSP in the NAc, galanin was applied to slices from GalR1 and GalR2 KO mice. Galanin had no effect on EPSP amplitude in the NAc in the absence of GalR1 or GalR2. ANOVA showed no effect of time or treatment in slices from GalR1 KO mice ($F_{4,32}$ = 0.9825, P = 0.4309, and $F_{1,8}$ = 0.2345, P = 0.6412; n = 5 cells from three female mice and two male mice; Fig. 7) or GalR2 KO mice ($F_{4,40}$ = 1.497, P = 0.2214, and $F_{1,10}$ = 0.3637, P = 0.5599; n = 6 cells from two female mice and four male mice; Fig. 8). There was an interaction of time and treatment in GalR1 KO recordings ($F_{4,32}$ = 4.775, P = 0.0039). No sex differences were observed, so data were pooled for subsequent analyses. Although the effects of galanin on EPSP amplitude do not wash out, as is often the case with large peptides in brain slices, the lack of effect of galanin in slices from GalR1 KO and GalR2 KO slices suggests that the effect of galanin on EPSP amplitude in WT slices is not attributable to run-down in these cells. These data suggest that signaling through both GalR1 and GalR2 is required for galanin-mediated suppression of glutamatergic excitation of MSNs.

CPP

To examine whether the GalR subtypes required for the physiological effects of galanin are also important in a behavior dependent upon the activity of the NAc, GalR1 and GalR2 KO mice and their WT littermate controls were tested for morphine CPP (3 and 5 mg/kg). The groups were composed as follows: WT GalR1 saline, n = 7 (four male mice and three female mice); WT GalR1 3 mg/kg, n = 9 (four male mice and five female mice); WT GalR1 5 mg/kg, n = 8 (four male mice and four female mice); GalR1 KO saline, n = 11 (three male mice and eight female mice); GalR1 KO 3 mg/kg, n = 11 (four male mice and seven female mice); GalR1 KO 5 mg/kg, n = 12 (five male mice and seven female mice); WT GalR2 saline, n = 14 (seven male mice and seven female mice); WT GalR2 3 mg/kg, n = 14 (six male mice and eight female mice); WT GalR2 5 mg/kg, n = 15 (eight male mice and seven female mice); GalR2 KO saline, n = 12 (five male mice and seven female mice); GalR2 KO 3 mg/kg, n = 14 (five male mice and nine female mice); and GalR2 KO 5 mg/kg, n = 12 (four male mice and eight

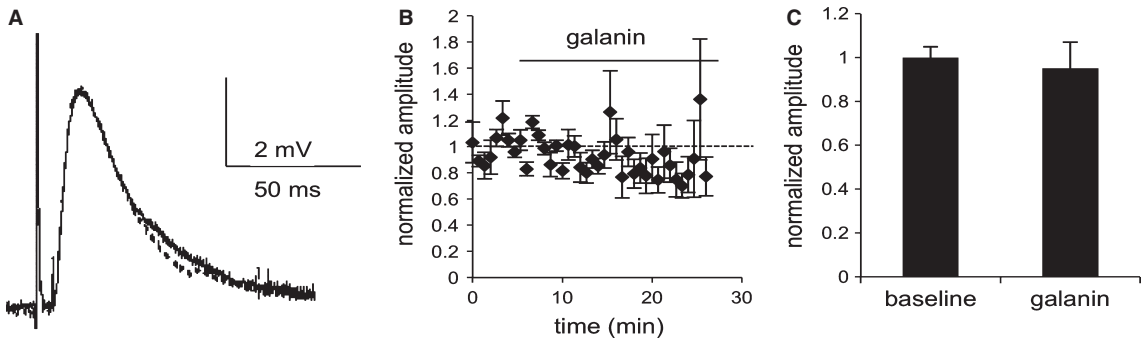

FIG. 7. MSNs in the NAc of GalR1 KO mice show no change in EPSP amplitude after application of galanin (1 μM). as (A) The average of five consecutive traces during the baseline (solid line) and galanin (dashed line) epochs of a representative experiment. (B) The combined normalised amplitudes (n = 5 cells from two male mice and three female mice) before and after galanin (1 μM) application at t = 0. (C) The mean of the averaged last five responses of the baseline and galanin epochs. Data are expressed as mean ± standard error (SE) of the mean.

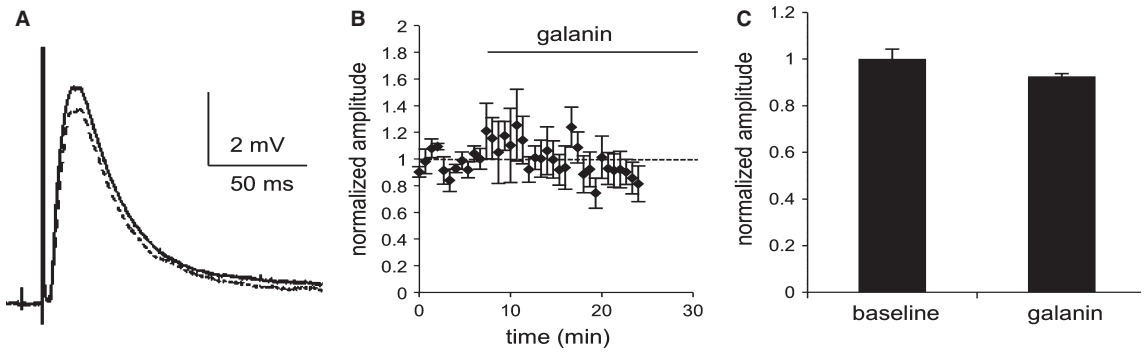

FIG. 8. MSNs in the NAc of GalR2 KO mice show no change in EPSP amplitude after application of galanin (1 μM). (A) The average of five consecutive traces during the baseline (solid line) and galanin (dashed line) epochs of a representative experiment. (B) The combined normalised amplitudes (n = 6 cells from four male mice and two female mice) before and after galanin (1 μM) application at t = 0. (C) The mean of the averaged last five responses of the baseline and galanin epochs. Data are expressed as mean ± standard error (SE) of the mean.

female mice). ANOVA revealed a main effect of genotype ($F_{1,51}$ = 4.952, P = 0.0305; Fig. 9) in the GalR1 KO experiment, and main effects of treatment ($F_{2,70}$ = 5.728, P = 0.0050; Fig. 9) and genotype ($F_{1,70}$ = 6.278, P = 0.0146) in the GalR2 KO experiment. *Post hoc* analyses indicated that WT GalR2 mice developed a significant preference for the morphine-paired chamber, whereas the GalR2 KO mice did not. The WT GalR1 mice showed a significantly increased preference for the morphine-paired chamber as compared with the GalR1 KO mice at the 3 mg/kg dose (P = 0.016), and an almost significant increase at the 5 mg/kg dose (P = 0.056). Analysis of post-test drug-paired chamber time including sex as a factor revealed a three-way treatment by genotype by sex interaction ($F_{2,112}$ = 3.8, P = 0.0253). This effect was driven by a somewhat larger difference between genotypes at 3 mg/kg morphine in female mice and at 5 mg/kg morphine in male mice; as the directionality of the KO effect was the same in males and females, data were pooled for subsequent analyses. These data demonstrate that neither GalR1 KO nor GalR2 KO mice respond to morphine in the same way as their WT littermates do.

Discussion

These NAc and DS recordings provide the first evidence that galanin acts directly on neurons whose activity is critical for the integration of reward signals (Carlezon & Thomas, 2009; Stuber *et al.*, 2012). Application of the galanin peptide reduces both field potentials and individual MSN synaptic responses to electrical stimulation. The inclusion of GABA receptor antagonists in the recording solution and the abolition of the signal in the presence of the AMPA receptor blocker 6-cyano-7-nitroquinoxaline-2,3-dione (Fig. S1) indicate that the reduction in fEPSPs could be representative of reduced glutamate signaling, although changes in cellular input resistance or cellular depolarisation cannot be ruled out. The ability of galanin to reduce EPSPs is absent in MSNs in the NAc of GalR1 and GalR2 KO mice. This indicates that both of these GalR subtypes are necessary for galanin modulation of glutamatergic input to the NAc. It is worth noting that GalR3 manipulations were not included in our study; however, although GalR3 is expressed in brain, it is more abundant in the periphery (Smith *et al.*, 1998), suggesting that it may contribute less to these brain mechanisms than GalR1 and GalR2. Interestingly, galanin acting through GalR1 and GalR2 to reduce striatal EPSPs has the potential to lead to either enhancement or dampening of drug reward as measured by CPP. The reduction in EPSP amplitude decreases the likelihood of NAc cell firing, potentially supporting reward processes (such as CPP), consistent with the theory that reduced NAc activity disinhibits downstream reward signaling via the ventral pallidum (Carlezon & Thomas, 2009). However, reduction in EPSPs could also result in reduced afferent connection to the prefrontal cortex, amygdala, and hippocampus, potentially reducing cue-related processes (such as CPP) via the uncoupling of affective, cognitive and spatial information from DA signaling (O'Donnell *et al.*, 1999b). It should be noted that a direct comparison between the behavioral and electrophysiological results presented here is complicated by the difference in mouse ages at the

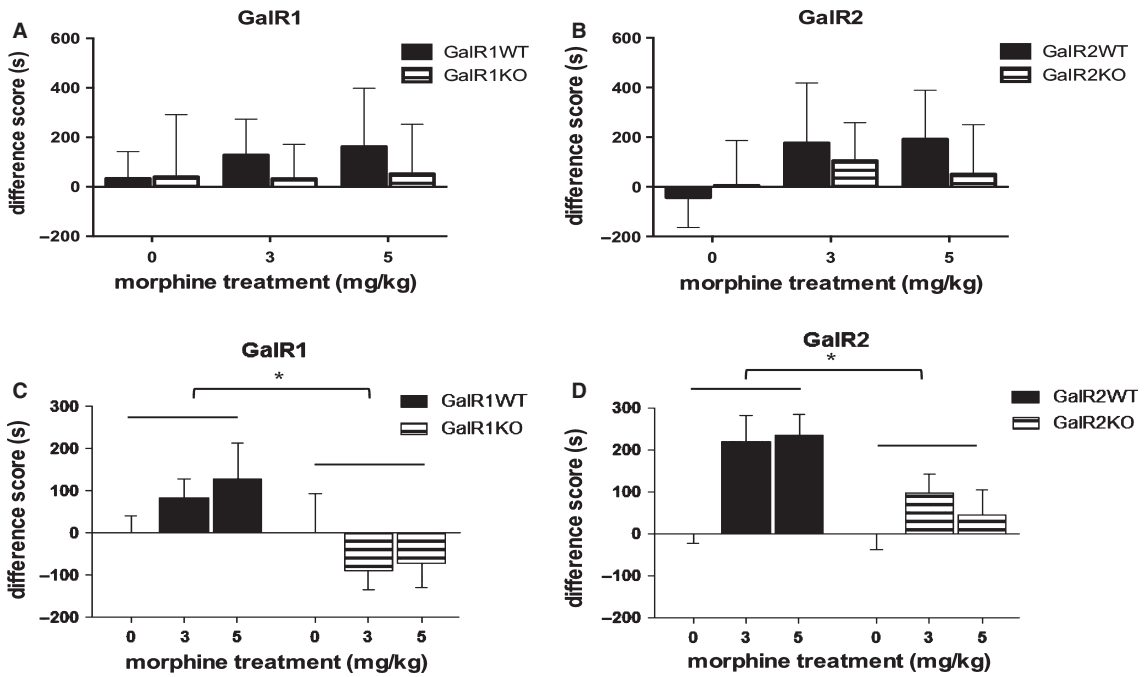

FIG. 9. Raw GalR1 (A) and GalR2 (B) difference scores and difference scores normalised to saline (C and D) demonstrate that GalR1 and GalR2 KO mice do not show significant morphine CPP at 3 or 5 mg/kg. Data are expressed as mean difference score ± standard error (SE) of the mean. $*P < 0.05$.

time of testing, although the ages of the mice used in the current studies are standard for the respective experimental paradigms (Narasimhaiah *et al.*, 2009; Higley *et al.*, 2011; Neugebauer *et al.*, 2011).

In contrast to what has been seen in mice lacking the galanin peptide, mice lacking either GalR1 or GalR2 showed greatly decreased morphine CPP. Although all of these studies confirm a role for the galanin system in morphine reward processing, the discrepancies in directionality are probably accounted for by differences in experimental approach. Evidence for a galanin-induced reduction in morphine conditioning comes from a study in which the galanin peptide was infused intracerebroventricularly (Zachariou *et al.*, 1999). Extensive studies have suggested that peptides with limited lipid solubility, such as galanin, will diffuse from a ventricular injection site in a manner that is strongly limited to the ventricular system (Yan *et al.*, 1994; Pardridge, 1997; Francis *et al.*, 2006). This indicates that an intracerebroventricular galanin infusion would act on galanin targets associated with the ventricular system, such as the locus coeruleus (Parker *et al.*, 1995; Kolakowski *et al.*, 1998; O'Donnell *et al.*, 1999a). Indeed, a reduction in morphine reward as a result of galanin activity in the locus coeruleus would be consistent with the idea that galanin reduces tonic locus coeruleus activity that encodes the presence of rewarding stimuli (Grenhoff *et al.*, 1993; Pieribone *et al.*, 1995; Ma *et al.*, 2001; Aston-Jones & Cohen, 2005).

A study of the effects of galanin peptide knockout in mice on a 129 Ola/Hsd background demonstrated that these animals showed normal morphine CPP at 3 and 5 mg/kg and were somewhat more sensitive to a very low dose of morphine (0.25 mg/kg; Hawes *et al.*, 2008). Although strain differences could explain an altered dose–response relationship for morphine, the fact that galanin peptide KO has different effects from KO of individual receptor subtypes suggests that the balance of signaling between GalR subtypes is likely to be important for the overall effect of galanin signaling in particular brain areas. One possibility that could explain the unexpected observation that GalR1 and GalR2 KO mice show reduced morphine CPP is that GalR1 can heterodimerise with D_1 and D_5 receptors (Moreno *et al.*, 2011). This interaction alters the signaling downstream of GalR1, reversing the effect of galanin from inhibitory to excitatory (Moreno *et al.*, 2011). Removal of one GalR subtype could therefore cause a reversal in signaling that would mimic the removal of the galanin peptide. Although the dimerisation with D_1 and D_5 receptors was only shown for GalR1, it is possible that this type of dimerisation is critical for GalR signaling in general, and that GalR2 may also dimerise with particular receptors, potentially altering its signaling.

Another consideration is that galanin peptide KO mice lack all products of the galanin precursor protein, including the galanin-like peptide (Wynick *et al.*, 1998), removing any signaling through GalRs. Such thorough disruption of the galanin system has been shown to result in compensatory signaling through neuropeptide Y (Hohmann *et al.*, 2003, 2004), which limits the interpretation of these results in the context of galanin alone. The GalR1 and GalR2 KO mouse strains contain deletions of only one receptor subtype, leaving the other receptors and the peptide itself intact (Jacoby *et al.*, 2002; Hobson *et al.*, 2006). This more targeted disruption of galanin signaling clearly demonstrates that expression of both GalR1 and GalR2 are required for a normal conditioning response to morphine.

The importance of galanin signaling to morphine reward suggests that galanin acts on reward circuitry, and previous studies provide additional evidence to support this idea. Galanin modulates responses to many drugs of abuse, including cocaine (Narasimhaiah *et al.*, 2009), amphetamine (Kuteeva *et al.*, 2005), alcohol (Karatayev *et al.*, 2009, 2010), and nicotine (Neugebauer *et al.*, 2011). Mice lacking the gene for galanin show increased sensitivity to cocaine reward as measured by CPP (Narasimhaiah *et al.*, 2009), although not as measured by self-administration (Brabant *et al.*, 2010), and decreased sensitivity to nicotine reward (Neugebauer *et al.*, 2011). Mice with elevated levels of galanin show an attenuated locomotor response to amphetamine (Kuteeva *et al.*, 2005) and higher levels of alcohol consumption (Karatayev *et al.*, 2009; McNamara & Robinson, 2010), whereas the

peptide KO mice show reduced levels of alcohol consumption (Karatayev *et al.*, 2010). These studies demonstrate that galanin can both attenuate (morphine, cocaine, and amphetamine) and potentiate (nicotine and ethanol) drug reward. Although central galanin infusion has been shown to increase dopamine synthesis in the forebrain and striatum as a response to decreased dopaminergic tone (Ericson & Ahlenius, 1999), and galanin reduces dopamine release in striatal slices (Tsuda *et al.*, 1998), the varied directionality of galanin's effects on reward behaviors suggests a more complicated role for the peptide in reward circuitry than the straightforward reduction of dopamine release.

Our electrophysiological data suggest a nuanced role for galanin in reward circuitry, as has been suggested previously by the varied directionality of galanin's effects on behaviors associated with drug reward. We have provided the first evidence that galanin acts directly on neurons of the mesocorticolimbic system, and have shown that its effects require the presence of both GalR1 and GalR2. In addition, we have shown that both of these receptor subtypes are required for morphine CPP. Increased understanding of the modulatory effects of peptides such as galanin on the cellular mechanisms of reward is vital for the future development of interventions to protect against the addictive properties of opioids.

Supporting Information

Additional supporting information can be found in the online version of this article:
Fig. S1. Placement of stimulating and recording electrodes in DS and NAc recordings.

Acknowledgements

The authors would like to thank Nadia Gavrilova and Samantha Sheppard for technical assistance with this project, and Dr Yann Mineur for helpful conversations. This work was supported by grant DA15425 from the National Institutes of Health and the State of Connecticut Department of Mental Health and Addiction Services (MRP), and by the Smith Family Foundation (MJH). The authors affirm that they have no conflicts of interest related to this work.

Abbreviations

ACSF, artificial cerebrospinal fluid; CPP, conditioned place preference; DS, dorsal striatum; EPSP, excitatory postsynaptic potential; fEPSP, excitatory postsynaptic field potential; GalR, galanin receptor; KO, knockout; MSN, medium spiny neuron; NAc, nucleus accumbens; WT, wild-type.

References

Aston-Jones, G. & Cohen, J.D. (2005) An integrative theory of locus coeruleus-norepinephrine function: adaptive gain and optimal performance. *Annu. Rev. Neurosci.*, **28**, 403–450.

Brabant, C., Kuschpel, A.S. & Picciotto, M.R. (2010) Locomotion and self-administration induced by cocaine in 129/OlaHsd mice lacking galanin. *Behav. Neurosci.*, **124**, 828–838.

Branchek, T.A., Smith, K.E., Gerald, C. & Walker, M.W. (2000) Galanin receptor subtypes. *Trends Pharmacol. Sci.*, **21**, 109–117.

Carlezon, W.A. Jr. & Thomas, M.J. (2009) Biological substrates of reward and aversion: a nucleus accumbens activity hypothesis. *Neuropharmacology*, **56**(Suppl 1), 122–132.

Carlezon, W.A. Jr. & Wise, R.A. (1996) Rewarding actions of phencyclidine and related drugs in nucleus accumbens shell and frontal cortex. *J. Neurosci.*, **16**, 3112–3122.

Chao, J. & Nestler, E.J. (2004) Molecular neurobiology of drug addiction. *Annu. Rev. Med.*, **55**, 113–132.

Compton, W.M. & Volkow, N.D. (2006) Major increases in opioid analgesic abuse in the United States: concerns and strategies. *Drug Alcohol Depen.*, **81**, 103–107.

Ericson, E. & Ahlenius, S. (1999) Suggestive evidence for inhibitory effects of galanin on mesolimbic dopaminergic neurotransmission. *Brain Res.*, **822**, 200–209.

Francis, A.B., Pace, T.W.W., Ginsberg, A.B., Rubin, B.A. & Spencer, R.L. (2006) Limited brain diffusion of the glucocorticoid receptor agonist RU28362 following i.c.v. administration: implications for i.c.v. drug delivery and glucocorticoid negative feedback in the hypothalamic–pituitary–adrenal axis. *Neuroscience*, **141**, 1503–1515.

Grenhoff, J., Nisell, M., Ferré, S., Aston-Jones, G. & Svensson, T.H. (1993) Noradrenergic modulation of midbrain dopamine cell firing elicited by stimulation of the locus coeruleus in the rat. *J. Neural Transm. Gen. Sect.*, **93**, 11–25.

Hawes, J.J., Brunzell, D.H., Narasimhaiah, R., Langel, U., Wynick, D. & Picciotto, M.R. (2008) Galanin protects against behavioral and neurochemical correlates of opiate reward. *Neuropsychopharmacol.*, **33**, 1864–1873.

Higley, M.J., Gittis, A.H., Oldenburg, I.A., Balthasar, N., Seal, R.P., Edwards, R.H., Lowell, B.B., Kreitzer, A.C. & Sabatini, B.L. (2011) Cholinergic interneurons mediate fast VGluT3-dependent glutamatergic transmission in the striatum. *PLoS ONE*, **6**, e19155.

Hobson, S.-A., Holmes, F.E., Kerr, N.C.H., Pope, R.J.P. & Wynick, D. (2006) Mice deficient for galanin receptor 2 have decreased neurite outgrowth from adult sensory neurons and impaired pain-like behaviour. *J. Neurochem.*, **99**, 1000–1010.

Hohmann, J.G., Krasnow, S.M., Teklemichael, D.N., Clifton, D.K., Wynick, D. & Steiner, R.A. (2003) Neuroendocrine profiles in galanin-overexpressing and knockout mice. *Neuroendocrinology*, **77**, 354–366.

Hohmann, J.G., Teklemichael, D.N., Weinshenker, D., Wynick, D., Clifton, D.K. & Steiner, R.A. (2004) Obesity and endocrine dysfunction in mice with deletions of both neuropeptide Y and galanin. *Mol. Cell. Biol.*, **24**, 2978–2985.

Hua, X.-Y., Hayes, C.S., Hofer, A., Fitzsimmons, B., Kilk, K., Langel, U., Bartfai, T. & Yaksh, T.L. (2004) Galanin acts at GalR1 receptors in spinal antinociception: synergy with morphine and AP-5. *J. Pharmacol. Exp. Ther.*, **308**, 574–582.

Hulse, R.P., Wynick, D. & Donaldson, L.F. (2011) Activation of the galanin receptor 2 in the periphery reverses nerve injury-induced allodynia. *Mol. Pain*, **7**, 26.

Jacoby, A.S., Hort, Y.J., Constantinescu, G., Shine, J. & Iismaa, T.P. (2002) Critical role for GALR1 galanin receptor in galanin regulation of neuroendocrine function and seizure activity. *Brain Res. Mol. Brain Res.*, **107**, 195–200.

Karatayev, O., Baylan, J. & Leibowitz, S.F. (2009) Increased intake of ethanol and dietary fat in galanin overexpressing mice. *Alcohol*, **43**, 571–580.

Karatayev, O., Baylan, J., Weed, V., Chang, S., Wynick, D. & Leibowitz, S.F. (2010) Galanin knockout mice show disturbances in ethanol consumption and expression of hypothalamic peptides that stimulate ethanol intake. *Alcohol. Clin. Exp. Res.*, **34**, 72–80.

Kolakowski, L.F. Jr., O'Neill, G.P., Howard, A.D., Broussard, S.R., Sullivan, K.A., Feighner, S.D., Sawzdargo, M., Nguyen, T., Kargman, S., Shiao, L.L., Hreniuk, D.L., Tan, C.P., Evans, J., Abramovitz, M., Chateauneuf, A., Coulombe, N., Ng, G., Johnson, M.P., Tharian, A., Khoshbouei, H., George, S.R., Smith, R.G. & O'Dowd, B.F. (1998) Molecular characterization and expression of cloned human galanin receptors GALR2 and GALR3. *J. Neurochem.*, **71**, 2239–2251.

Koob, G.F. (1992) Neural mechanisms of drug reinforcement. *Ann. NY Acad. Sci.*, **654**, 171–191.

Koob, G.F. & Volkow, N.D. (2010) Neurocircuitry of addiction. *Neuropsychopharmacol.*, **35**, 217–238.

Kuehn, B.M. (2007) Opioid prescriptions soar: increase in legitimate use as well as abuse. *JAMA*, **297**, 249–251.

Kuteeva, E., Hökfelt, T. & Ogren, S.O. (2005) Behavioural characterisation of transgenic mice overexpressing galanin under the PDGF-B promoter. *Neuropeptides*, **39**, 299–304.

Lang, R., Gundlach, A.L. & Kofler, B. (2007) The galanin peptide family: receptor pharmacology, pleiotropic biological actions, and implications in health and disease. *Pharmacol. Ther.*, **115**, 177–207.

Lovinger, D.M., Tyler, E., Fidler, S. & Merritt, A. (1993) Properties of a presynaptic metabotropic glutamate receptor in rat neostriatal slices. *J. Neurophysiol.*, **69**, 1236–1244.

Ma, X., Tong, Y.G., Schmidt, R., Brown, W., Payza, K., Hodzic, L., Pou, C., Godbout, C., Hökfelt, T. & Xu, Z.Q. (2001) Effects of galanin receptor agonists on locus coeruleus neurons. *Brain Res.*, **919**, 169–174.

Matthews, R.T. & German, D.C. (1984) Electrophysiological evidence for excitation of rat ventral tegmental area dopamine neurons by morphine. *Neuroscience*, **11**, 617–625.

McNamara, I.M. & Robinson, J.K. (2010) Conditional stimulation by galanin of saccharin and ethanol consumption under free and response contingent access. *Neuropeptides*, **44**, 445–451.

Moreno, E., Vaz, S.H., Cai, N.-S., Ferrada, C., Quiroz, C., Barodia, S.K., Kabbani, N., Canela, E.I., McCormick, P.J., Lluis, C., Franco, R., Ribeiro, J.A., Sebastiao, A.M. & Ferre, S. (2011) Dopamine–galanin receptor heteromers modulate cholinergic neurotransmission in the rat ventral hippocampus. *J. Neurosci.*, **31**, 7412–7423.

Narasimhaiah, R., Kamens, H.M. & Picciotto, M.R. (2009) Effects of galanin on cocaine-mediated conditioned place preference and ERK signaling in mice. *Psychopharmacology*, **204**, 95–102.

Neugebauer, N.M., Henehan, R.M., Hales, C.A. & Picciotto, M.R. (2011) Mice lacking the galanin gene show decreased sensitivity to nicotine conditioned place preference. *Pharmacol. Biochem. Behav.*, **98**, 87–93.

O'Donnell, D., Ahmad, S., Wahlestedt, C. & Walker, P. (1999a) Expression of the novel galanin receptor subtype GALR2 in the adult rat CNS: distinct distribution from GALR1. *J. Comp. Neurol.*, **409**, 469–481.

O'Donnell, P., Greene, J., Pabello, N., Lewis, B.L. & Grace, A.A. (1999b) Modulation of cell firing in the nucleus accumbens. *Ann. NY Acad. Sci.*, **877**, 157–175.

Pardridge, W.M. (1997) Drug delivery to the brain. *J. Cereb. Blood Flow Metab.*, **17**, 713–731.

Parker, E.M., Izzarelli, D.G., Nowak, H.P., Mahle, C.D., Iben, L.G., Wang, J. & Goldstein, M.E. (1995) Cloning and characterization of the rat GALR1 galanin receptor from Rin14B insulinoma cells. *Brain Res. Mol. Brain Res.*, **34**, 179–189.

Picciotto, M.R., Brabant, C., Einstein, E.B., Kamens, H.M. & Neugebauer, N.M. (2010) Effects of galanin on monoaminergic systems and HPA axis: potential mechanisms underlying the effects of galanin on addiction- and stress-related behaviors. *Brain Res.*, **1314**, 206–218.

Pieribone, V.A., Xu, Z.Q., Zhang, X., Grillner, S., Bartfai, T. & Hökfelt, T. (1995) Galanin induces a hyperpolarization of norepinephrine-containing locus coeruleus neurons in the brainstem slice. *Neuroscience*, **64**, 861–874.

Przewłocka, B., Machelska, H., Rekowski, P., Kupryszewski, G. & Przewłocki, R. (1995) Intracerebroventricular galanin and N-terminal galanin fragment enhance the morphine-induced analgesia in the rat. *J. Neural Transm. Gen. Sect.*, **102**, 229–235.

Saar, K., Mazarati, A.M., Mahlapuu, R., Hallnemo, G., Soomets, U., Kilk, K., Hellberg, S., Pooga, M., Tolf, B.-R., Shi, T.S., Hökfelt, T., Wasterlain, C., Bartfai, T. & Langel, U. (2002) Anticonvulsant activity of a nonpeptide galanin receptor agonist. *Proc. Natl. Acad. Sci. USA*, **99**, 7136–7141.

Sergeeva, O.A., Chepkova, A.N., Doreulee, N., Eriksson, K.S., Poelchen, W., Monnighoff, I., Heller-Stilb, B., Warskulat, U., Haussinger, D. & Haas, H.L. (2003) Taurine-induced long-lasting enhancement of synaptic transmission in mice: role of transporters. *J. Physiol.*, **550**, 911–919.

Shippenberg, T.S. & Elmer, G.I. (1998) The neurobiology of opiate reinforcement. *Crit. Rev. Neurobiol.*, **12**, 267–303.

Smith, K.E., Forray, C., Walker, M.W., Jones, K.A., Tamm, J.A., Bard, J., Branchek, T.A., Linemeyer, D.L. & Gerald, C. (1997) Expression cloning of a rat hypothalamic galanin receptor coupled to phosphoinositide turnover. *J. Biol. Chem.*, **272**, 24612–24616.

Smith, K.E., Walker, M.W., Artymyshyn, R., Bard, J., Borowsky, B., Tamm, J.A., Yao, W.J., Vaysse, P.J., Branchek, T.A., Gerald, C. & Jones, K.A. (1998) Cloned human and rat galanin GALR3 receptors. Pharmacology and activation of G-protein inwardly rectifying K^+ channels. *J. Biol. Chem.*, **273**, 23321–23326.

Stuber, G.D., Britt, J.P. & Bonci, A. (2012) Optogenetic modulation of neural circuits that underlie reward seeking. *Biol. Psychiat.*, **71**, 1061–1067.

Sun, Y.-G. & Yu, L.-C. (2005) Interactions of galanin and opioids in nociceptive modulation in the arcuate nucleus of hypothalamus in rats. *Regul. Peptides*, **124**, 37–43.

Tsuda, K., Tsuda, S., Nishio, I., Masuyama, Y. & Goldstein, M. (1998) Effects of galanin on dopamine release in the central nervous system of normotensive and spontaneously hypertensive rats. *Am. J. Hypertens.*, **11**, 1475–1479.

Wiesenfeld-Hallin, Z., Xu, X.J., Villar, M.J. & Hökfelt, T. (1990) Intrathecal galanin potentiates the spinal analgesic effect of morphine: electrophysiological and behavioural studies. *Neurosci. Lett.*, **109**, 217–221.

Wynick, D., Small, C.J., Bacon, A., Holmes, F.E., Norman, M., Ormandy, C.J., Kilic, E., Kerr, N.C., Ghatei, M., Talamantes, F., Bloom, S.R. & Pachnis, V. (1998) Galanin regulates prolactin release and lactotroph proliferation. *Proc. Natl. Acad. Sci. USA*, **95**, 12671–12676.

Xu, Z.-Q.D., Zheng, K. & Hökfelt, T. (2005) Electrophysiological studies on galanin effects in brain – progress during the last six years. *Neuropeptides*, **39**, 269–275.

Yan, Q., Matheson, C., Sun, J., Radeke, M.J., Feinstein, S.C. & Miller, J.A. (1994) Distribution of intracerebral ventricularly administered neurotrophins in rat brain and its correlation with trk receptor expression. *Exp. Neurol.*, **127**, 23–36.

Zachariou, V., Parikh, K. & Picciotto, M.R. (1999) Centrally administered galanin blocks morphine place preference in the mouse. *Brain Res.*, **831**, 33–42.

Zhang, Y.P., Lundeberg, T. & Yu, L.C. (2000) Interactions of galanin and morphine in the spinal antinociception in rats with mononeuropathy. *Brain Res.*, **852**, 485–487.