Galanin Acts at GalR1 Receptors in Spinal Antinociception: Synergy with Morphine and AP-5

Xiao-Ying Hua
Carol S. Hayes
Anthony Hofer
Bethany Fitzsimmons
Kalle Kilk
Ülo Langel
Tamas Bartfai
Tony L. Yaksh

Department of Anesthesiology, University of California San Diego, La Jolla, CA (X.-Y. H., C.S.H., A.H., B.F. and T.L.Y.)

Department of Neuropharmacology, The Harold Dorris Neurological Institute, The Scripps Research Institute, La Jolla, CA (K.K., Ü.L. and T.B.)
Running title: Spinal antinociception of galanin and analogues

Corresponding author: Xiao-Ying Hua, Ph.D., Anesthesia Research Laboratory, 0818, Department of Anesthesiology, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92103-0818.
Tel: (619) 543-3597; Fax: (619) 543-6070; E-mail: xyhua@ucsd.edu

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Abbreviations: AP-5 (2-amino-5-phosphonopentanoic acid), DRG (Dorsal root ganglia), Gal (Galanin), GalR (Galanin receptor), GALP (Galanin-like peptide), IT (intrathecal), NMDA (N-methyl-D-aspartate), NO (Nitric oxide), SP (Substance P).

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Abstract

The neuropeptide galanin and its receptors (GalR1, GalR2 and GalR3) are expressed in spinal cord. We have characterized the pharmacology of the antinociceptive effects of intrathecally (IT) administered galanin and its analogues in the formalin test in rats, using an automated flinch detection system. Intrathecal injection of rat galanin (Gal1-29) or human galanin (Gal1-30) produced a dose-dependent inhibition of formalin-evoked flinching in phase 2, but not in phase 1. Relative potency of galanin homologues is Gal1-29 ≥ Gal1-30 > Galanin-like peptide1-24 (GALP1-24) ≥ Gal2-11 = Gal 3-29 (an inactive analog). Galanin1-29 and Gal1-30 are both high affinity agonists to GalR1/R2, while Gal2-11 is a GalR2 receptor agonist. Our data suggest that IT galanin produced antinociception is mediated by activation of GalR1 receptors. When comparing antinociceptive effects of IT Gal1-29 to morphine and to 2-amino-5-phosphonopentanoic acid (AP-5, an NMDA antagonist), Gal1-29 is of intermediate potency between these two analgesic agents based on the ED50 values. An isobolographic analysis showed synergy between Gal1-29 and morphine and between Gal1-29 and AP-5 on the 2nd phase. Fixed-ratio dose combinations of morphine and Gal1-29, or AP-5 and Gal1-29 produced significantly greater antinociception than predicted from simple additivity. In summary, the present findings reveal that (1) spinal galanin produces a reliable inhibition of formalin-induced facilitated nociceptive processing, an effect possibly mediated by GalR1 receptors, and (2) galanin potentiates IT morphine and AP-5 induced antinociception.
The neuropeptide galanin is expressed in dorsal root ganglia (DRG) and spinal cord (Hokfelt et al., 1987). About half of the galanin-positive terminals in the dorsal horn are in primary afferent fibers (Zhang et al., 1993; Zhang et al., 1995a), and a considerable amount of galanin is present in a subpopulation of lamina II interneurons, which also contain GABA and enkephalins (Simmons et al., 1995; Zhang et al., 1995b). Three galanin receptor subtypes, named GalR1, GalR2 and GalR3, have been cloned, and belong to the superfamily of the G-protein coupled receptors (see (Branchek et al., 2000)). Activation of either GalR1 or GalR3 produces hyperpolarization via $G_{i/o}$ and inhibits adenylyl cyclase. GalR2 activation leads to stimulation of phospholipase C via $G_{q/11}$, producing calcium mobilization, diacylglycerol formation and subsequent activation of protein kinase C (see (Branchek et al., 2000)). All three receptor transcripts are present in DRG and spinal cord (Waters and Krause, 2000). GalR1 mRNA has been found in lamina II local neurons (Parker et al., 1995). The anatomical location of galanin and galanin receptors in DRG and spinal cord suggests that endogenous or exogenous galanin may participate in the regulation of spinal nociceptive transmission.

Spinal effects of galanin on nociception appear complex, as both facilitatory and inhibitory effects have been observed. Early studies showed that IT galanin at low doses ($\leq 1$ nmol) elicits a facilitation of the flexor reflex in rats (Wiesenfeld-Hallin et al., 1988; Wiesenfeld-Hallin et al., 1989; Xu et al., 1990), and increases responsiveness to noxious stimulation (Cridland and Henry, 1988). Recent studies on transgenic mice null mutated for galanin peptide demonstrated that the lack of galanin expression attenuates both spontaneous and stimulation-induced pain behaviors following peripheral nerve injury and inflammation (Kerr et al., 2000;
Kerr et al., 2001). Conversely, expression of galanin is upregulated in sensory neurons and spinal cord after nerve injury where hyperalgesia and allodynia are observed (Hokfelt et al., 1987). This suggests that the increased level of spinal galanin may contribute to pain behavior. Although there are no selective galanin receptor antagonists, the excitatory effect of galanin is apparently mediated by activation of the GalR2 receptor (Liu et al., 2001). An inhibitory action of IT galanin on spinal nociceptive processing is also reported. This effect usually requires a higher dose, and is presumably mediated through activation of the GalR1 (see (Xu et al., 2000; Liu and Hokfelt, 2002)). In agreement with the antinociceptive effects of spinal galanin, electrophysiological studies reveal that galanin attenuates C-fiber stimulation induced nociceptive reflexes and dorsal horn neuron hyperexcitability (Yanagisawa et al., 1986). There are apparently some interactions between galanin and opioids on spinal antinociception. Spinally administrated M15 and M35, two chimeric peptide-type putative galanin receptor antagonists, attenuate analgesic actions of intrathecal morphine and DAMGO (Reimann et al., 1994), suggesting that endogenous galanin may participate in spinal opioid-induced analgesia. Zhang et al. (Zhang et al., 2000), however, reported that IT galanin induced a naloxone reversible increase in paw withdrawal latency to thermal and mechanical stimulation, suggesting that galanin facilitates a release of endogenous opioids in spinal cord. Inhibition induced by morphine and an antagonist of cholecystokinin B receptor on spinal nociceptive reflexes is enhanced by galanin (Wiesenfeld-Hallin et al., 1990).

Animal behaviors used to assess antinociceptive effects of spinal galanin typically are measurement of paw escape latencies and thresholds to thermal and mechanical stimulation, respectively. In the present work, we undertook a series of experiments in rats using the
automated formalin test device (Yaksh et al., 2001) to characterize (1) the antinociceptive effects of IT administration of galanin (i.e., Gal\textsubscript{1-29}) and its analogues, which have different affinity for GalR1 and GalR2 receptors; and (2) the synergy between Gal\textsubscript{1-29} and two analgesic agents, i.e., morphine and AP-5, by the use of an isobolographic analysis.
METHODS

Animals

Male Holzman Sprague Dawley rats (320-360g, Harlan Sprague Dawley, Indianapolis, IN) were used in accordance with protocols approved by the Animal Care Committee of the University of California. The animals were maintained in a group colony on an ad libitum diet and on a 12 hr day-12 hr night cycle.

Intrathecal implantation

Rats were implanted with a single-lumen intrathecal catheter (Yaksh and Rudy, 1976) for drug delivery. To place the catheter, anesthesia was induced with 4% isoflurane in a room air/oxygen mixture (1:1). The back of the head and neck were shaved, and the animals then were placed in a stereotaxic head holder with the head flexed forward. Anesthesia was maintained with 2% isoflurane delivered by mask. A midline incision was made on the back of the neck. The muscles were freed at the attachment to the skull exposing the cisternal membrane. A small (~1mm) puncture was made in the dura, and an 8.5 cm polyethylene (PE-10) catheter was then inserted through the cisternal opening, and carefully passed caudally into the intrathecal space terminating at the L1-3 spinal segments. The end of the catheter was tunneled subcutaneously over the front dorsal skull bones, flushed with 10μl saline, and then plugged with a short length of wire. The skin incision was then closed using 3.0 USP black braided silk suture. The rats were given 5ml of Lactated Ringer’s solution subcutaneously and allowed to recover under a heat lamp. Rats showing motor weakness or signs of paresis upon recovery from anesthesia were euthanized immediately. Animals recovered for 5-7 days prior to the formalin test.
**Intrathecal Drugs and Injection**

All drugs were injected IT in a total volume of 10µl followed by 10µl of saline to flush the catheter. The following drugs were used in this study: Galanin<sub>1-29</sub> (Gal<sub>1-29</sub>, MW: 3165), Galanin<sub>1-30</sub> (Gal<sub>1-30</sub>, MW: 3157), Galanin-like peptide<sub>1-24</sub> (GALP<sub>1-24</sub>, MW: 2500), Galanin<sub>2-11</sub> (Gal<sub>2-11</sub>, MW: 1137), Galanin<sub>3-29</sub> (Gal<sub>3-29</sub>, MW: 2920), Galanin<sub>1-13-Pro-Bradykinin2-9</sub> amide (M35, MW: 2249), all were synthesized on solid phase with tBOC chemistry and the HPLC purified peptides analyzed by mass spectrometry. A non-peptide ligand for galanin receptor: Fmoc-cycloxyxalanine-Lys-amidomethylcoumarin (Galnon, MW: 677) was synthesized as described by Saar et al (Saar et al., 2002). Morphine (morphine sulfate, MW: 669) was obtained from Merck, Sharp and Dohme (West Point, PA), and AP-5 (±-2-Amino-5-phosphonopentanoic acid, MW: 197) from RBI (Natrick, MA). A single IT drug injection was given 10 min prior to the formalin paw injection. For the drug combinations, Gal<sub>1-29</sub> was given at 20 min prior to, and morphine or AP-5 given at 10 min prior to the formalin paw injection. All the drug solutions except Galnon, which was in 10% DMSO, were made in physiological saline.

**Formalin test**

To quantify formalin paw injection induced flinching/licking behavior, an automated sensing system was employed (Yaksh et al., 2001). Briefly, a soft metal band (10mm wide and 27 mm long, 0.5g, C-shape) was placed on one of the hind paws of a rat. After acclimation for 30 min, animals were gently restrained and 50µl of 2.5% formalin solution was injected subcutaneously into the dorsal surface of the banded paw with a 30-guage needle. Data collection was initiated after the animal was placed inside of the test chamber. Nociceptive
behavior was quantified by automatically counting incidences of spontaneous flinching/shaking of the injected paw. The flinches were counted over 1-min intervals for 60 min. The animals were sacrificed with CO₂ immediately after the test.

Data Analysis and Statistics

Antinociceptive ED₅₀ values to the formalin test were calculated from the dose-response curves generated for galanin (Gal₁₋₂₉), galanin analogues, morphine and AP-5 alone or in combination based on the graded dose response method of Tallarida and Murray (Tallarida and Murray, 1987). Combination of the two drugs (i.e., Gal₁₋₂₉ + morphine, or Gal₁₋₂₉ + AP-5) was obtained in a constant dose ratio based on the ED₅₀ values of the single agents (1/2, 1/4 and 1/8 ED₅₀). Drug synergism was analyzed by the conventional isobolographic analysis (Tallarida et al., 1989). Briefly, ED₅₀ values of drugs alone were plotted, and a theoretical additive line constructed on an isobologram. Experimental values from fixed-ratio designed studies were also analyzed using linear regression, and an ED₅₀ values for each combination was determined and plotted on the isobologram for the comparison to the theoretical additive value. The Student’s t test was used to determine significance of the difference between the theoretical additive point and experimental derived ED₅₀ value. A P value less than 0.05 indicated that drugs produced a synergistic effect as compared to either drug by itself. A total fraction value that reveals what portion of the single ED₅₀ value was accounted for by the corresponding ED₅₀ value for the combination was also calculated. Values less than 1 indicate a multiplicative interaction. Total fractions were calculated as previously described by Roerig and Fujimoto (Roerig and Fujimoto, 1988):

\[
\frac{ED_{50 \text{ dose in combination of drug 1}}}{ED_{50 \text{ value for drug 1 given alone}}} + \frac{ED_{50 \text{ dose in combination of drug 2}}}{ED_{50 \text{ value for drug 2 given alone}}}
\]
All the data are presented as mean ± SEM. For the data in the Figures 1-3, statistical significance was calculated using one-way analysis of variance with multiple comparisons for independent measurement (ANOVA) followed by Dunnett’s test by using the Prism computer program. Differences were considered to be significant when the critical value reached a level of $P < 0.05$. 
RESULTS

As previously noted, formalin injected into the paw caused two phases of flinching behavior. Phase 1 started with initial intense flinches occurring 1-2 min post-injection, followed by a rapid decline in min 5-6. Phase 2 began after 15-20 min with the maximal response typically observed around 25-30 min after the formalin injection (Malmberg and Yaksh, 1992). Using the automated flinch detecting system, the response count and distribution resembles that obtained with the manual counting system (Yaksh et al., 2001). Thus, the biphasic display of paw flinch behavior evoked by formalin paw injection (2.5%, 50 µl) has been used in the present study to evaluate the antinociceptive activity of IT galanin and its analogues.

Galanin and analogues

In IT saline-treated rats, the total number of flinches after the formalin injection was 195±19 in phase 1 (1-9 min), and 1094±109 in phase 2 (10-60 min) of the response (N=9, Figure 1). These numbers are in the same range as that observed for naïve rats (Yaksh et al., 2001). Intrathecal injection of either rat galanin Gal1-29 or the human galanin Gal1-30, both high affinity agonists to GalR1 and GalR2 receptors (Ki =1 nM), produced a dose-dependent (3-30 nmol) inhibition of the phase 2 flinching response; phase 2A (10-40 min) was preferentially affected (Figures 1 and 2). In contrast, neither Gal1-29 nor Gal1-30 significantly altered flinching within phase 1 (Figures 1 and 2). Gal1-29 or Gal1-30 at the highest dose, i.e., 30 nmol, which did not produce any sign of motor weakness or motor disfunction, markedly reduced the 2nd phase response in comparison to the saline group (46–47% of saline group, N= 5-7, p < 0.05). Gal3-29, an N-terminally truncated inactive ligand (Ki > 1000 nM), given at the highest equivalent dose as Gal1-29 (30 nmol), did not significantly attenuate flinching behavior in either phase (N=4, Figure
3). GALP\textsubscript{1-24}, is a synthetic fragment of the recently discovered GALP (Ohtaki et al., 1999), which is a 60 amino acid long peptide isolated from porcine hypothalamus containing an internal sequence (amino acids 9-21) identical to the N-terminal sequence of galanin. GALP has a similar affinity as Gal\textsubscript{1-29} for GalR1 (IC\textsubscript{50} 2nM), but a slightly higher affinity for GalR2 (IC\textsubscript{50} 0.2nM). GALP\textsubscript{1-24} is a likely peptidolytic cleavage product of GALP. In the present study, GALP\textsubscript{1-24} at doses of 3 and 10 nmol had no effect (data not shown). Increased doses of GALP\textsubscript{1-24} (30nmol) produced 45\% inhibition of the 2\textsuperscript{nd} phase (Figure 3), however, this effect was not statistically significant. Gal\textsubscript{2-11}, with selective GalR2 agonist activity (IC\textsubscript{50}: 1.8nM for GalR2, and 879 nM for GalR1), at doses of 30nmol had little effect on the flinching response (N=5, Figure 3). The ED\textsubscript{50} values (nmol) of galanin, based on the inhibition of phase 2 are: Gal\textsubscript{1-29} 19 (95\% C.I., 8-47) and Gal\textsubscript{1-30} 30 (95\% C.I., 3-311). The estimated ED\textsubscript{50} value of GALP\textsubscript{1-24} is 48 (95\% C.I., 5-458). Since neither Gal\textsubscript{2-11} nor Gal\textsubscript{3-29} at 30 nmol displayed a significant inhibition of the flinching behavior, the ED\textsubscript{50} values of these two analogues, if there were any, would be estimated to be higher than 48 nmol. The rank order of potency of galanin homologues based on the ED\textsubscript{50} values (or the estimated ED\textsubscript{50} values) is Gal\textsubscript{1-29} \geq Gal\textsubscript{1-30} > GALP\textsubscript{1-24} \geq Gal\textsubscript{2-11} = Gal\textsubscript{3-29}.

Previous work has shown that spinal administration of galanin, typically at low doses (\leq 1nmol) also produces excitatory effects on nociception (see Introduction), however we saw no facilitatory activities of either galanin or the peptide analogues at all examined doses including the low dose (3nmol).

Galnon, a non-peptide agonist-like ligand of the galanin receptor with 1000 times lower affinity (Ki, 4.8\mu M) than Gal\textsubscript{1-29} for the GalR1 (Saar et al., 2002), given at a dose of 30 nmol IT did not alter the flinching response in either phase (N=12, Figure 3). A chimeric peptide of
galanin1-13 and bradykinin2-9, M35, has a similar affinity for GalR1 and GalR2 as Gal1-29 (Ki 1-10 nM), and have been shown to function as an antagonist/partial agonist (see (Xu et al., 2000)). Intrathecal administration of M35 to rats at four doses (i.e., 0.3, 1, 3 and 10nmol, N=4-8) did not potentiate flinching response (data not shown), while the highest dose (i.e., 10 nmol) reduced the 2nd phase flinching (M35: 549±160, Saline: 1021±100; p < 0.05). Pretreatment with M35 at 0.3-1 nmol alone showed little effect on formalin-induced flinching, and did not reverse the inhibitory effects of Gal1-29 or Gal1-30 (data not shown). No animal showed abnormal sensory or motor function after IT administration of galanin analogues, including Galnon and M35, at all examined doses.

**Interaction with morphine or AP-5**

Intrathecal injection of morphine (0.3-100 nmol, N=22) or AP-5 (5.6-152 nmol, N=16) resulted in a dose-dependent inhibition of 2nd phase flinching behavior (Figure 4, Table 1). AP-5 at the highest dose (152 nmol) also significantly reduced flinching within the 1st phase (data not shown). This result is in agreement with previous observations (Yamamoto and Yaksh, 1992; Yaksh et al., 2001). The relative potency of IT Gal1-29 as compared to these two analgesic agents (ED50, nmol, phase 2) is: Morphine (4) > Gal1-29 (19) > AP-5 (60) (Table 1). Synergistic antinociception between Gal1-29 and morphine or Gal1-29 and AP-5 in the formalin test was observed (Figures 5 and 6, Table 1). Isobolographic analysis of the fixed dose combination of Gal1-29 and morphine (N=11), or combination of Gal1-29 and AP-5 (N=11) on the 2nd phase indicated that the interaction is synergistic. Figures 5B and 6B show the plots of the combination ED50 values in relation to the ED50 values of the drugs alone. In the series of dose combinations that were examined using the fixed dose ratios for morphine and Gal1-29 (1:4.8), or
AP-5 and Gal1-29 (1:0.3), all the points fell below the line of additivity, and the differences are statistically significant ($p < 0.05$). The line of additivity reflects the theoretical dose combination required to produce 50% of the effect, assuming the agents interact in a linear fashion. Total dose fractions (2nd phase) were calculated, 0.54 for the combination of morphine and Gal1-29 and 0.56 for AP-5 and Gal1-29. The values near 1 indicate an additive effect, and values less than 1 imply a multiplicative interaction. No motor weakness or sensory disfunctions were seen in animals with the combination drug treatment.
DISCUSSION

In the present work, we demonstrated that the spinal administration of Gal$_{1-29}$ or Gal$_{1-30}$ in rats produced a reliable and robust inhibition (50-70\% reduction) of second phase flinching evoked by formalin paw injection. It is important to note that antinociceptive effects of both Gal$_{1-29}$ and Gal$_{1-30}$ were not associated with sedation or motor impairment. In comparison to the antihyperalgesic effects of morphine and AP-5, Gal$_{1-29}$ is of intermediate potency between these two classes of analgesic agents. Thus these experiments are in agreement with earlier work (see (Xu et al., 2000; Liu and Hokfelt, 2002)), and confirm the antinociceptive actions of spinal galanin.

Galanin and analogues

An aim of the present work was to assess the potential contribution of galanin receptor subtypes. Accordingly, we considered the dose-dependent inhibitory effects of several galanin analogues that have differential affinity for the GalR subtypes. Binding studies have shown that both Gal$_{1-29}$ and Gal$_{1-30}$ have high affinity for GalR1 and GalR2 receptors (K$_i$ values about 1nM), while Gal$_{3-29}$, has approximately 1000-fold lower affinity for all three galanin receptors (K$_i$ >1000nM). Consistent with the binding data, the present findings also revealed that IT Gal$_{3-29}$ displayed no effect on formalin-induced nociception, in contrast to the marked antinociception produced by IT Gal$_{1-29}$ and Gal$_{1-30}$. It has been shown that GALP$_{1-24}$ displaces $^{125}$I-galanin from galanin binding sites in mouse hippocampal membranes with high affinity (IC$_{50}$ 2nM), similar to that of Gal$_{1-29}$ (Ohtaki et al., 1999). However, the antinociceptive effect of GALP$_{1-24}$ on the formalin test appears weak (estimated ED$_{50}$ 48nmol). This suggests that galanin and GALP$_{1-24}$
may have different GalR subtype specificity, as we know that GALP has a higher affinity for GalR2 (IC\textsubscript{50} 0.2\text{nM}) than for GalR1 (IC\textsubscript{50} 4\text{nM}) (Ohtaki et al., 1999). The galanin fragment Gal\textsubscript{2-11}, which is 500-fold more selective for GalR2 (IC\textsubscript{50} 1.76 \text{nM}) over GalR1 (IC\textsubscript{50} 879 \text{nM}) (Liu et al., 2001, and a similar observation from our lab), displayed no effect on formalin-evoked nociceptive response. This is in agreement with the finding that IT infusion of Gal\textsubscript{2-11} in rats produced only hyperalgesia, and not analgesia (Liu et al., 2001). Taken together, our data support the assertion that the antinociceptive effect of IT galanin is mediated by activation of spinal GalR1, but not GalR2 receptors. We recognize the limitation of asserting this hypothesis regarding GalR1 vs. GalR2 effects based on the lack of effect of a single drug (i.e., Gal\textsubscript{2-11}). However, given the marked and effective binding affinity of Gal\textsubscript{2-11} for GalR2, the lack of effect of a dose of this agent equalmolar to the efficacious doses of Gal\textsubscript{1-29} and Gal\textsubscript{1-30} provides substantive support for our assertion. Development of additional GalR2 selective agonists will be required to confirm this hypothesis.

Galnon is a low molecular weight galanin agonist-like ligand, which can penetrate the blood-brain barrier, and has potent anticonvulsant activity (Saar et al., 2002). The K\textsubscript{i} value of Galnon at the human GalR1 receptor is 4.8\text{µM}. A recent study demonstrated that systemic galnon attenuated peripheral nerve injury induced thermal hyperalgesia in rats, and the effect was blocked by spinal M35 (Wu et al., 2003). In the present study, administration of Galnon directly into the intrathecal space at a dose of 30 nmol produced no inhibition of the formalin-induced flinching response. This discrepancy may suggest that the antihyperalgesic activity of systemic Galnon could be due to either release of endogenous galanin, or to an effect at GalRs outside the spinal cord.
Although M35 acts as partial agonist both *in vitro* and *in vivo* (see (Branchez et al., 2000; Xu et al., 2000)), its value lies in its utility as a GalR-specific antagonist. M35 behaves as an antagonist of exogenous galanin action in several regions of CNS (Bartfai et al., 1991; Branchek et al., 2000). Previous work has shown that the spinal effect of galanin is antagonized by M35 and other chimeric peptides (e.g., M15, M32) (Bartfai et al., 1991; Wiesenfeld-Hallin et al., 1992). In contrast, in the present study, M35 exerted an agonist-like action. M35 at 10 nmol significantly attenuated 2nd phase flinches. However, low doses of M35 (0.3-1 nmol) displayed little or no agonist activity, but did not reverse the antinociception of Gal1-29 or Gal1-30. These complex effects of M35 are not unexpected given that this molecule is a chimeric peptide with galanin1-13 as the N-terminal portion. This sequence defines the recognition of the ligand by galanin receptors and likely accounts for the agonist-like effect seen here. Although Wiesenfeld-Hallin and her colleagues (Wiesenfeld-Hallin et al., 1992) reported previously that intrathecal M35 blocks excitatory effects of low dose spinal galanin, we did not observe substantial stimulatory activity of IT Gal1-29 or Gal1-30 at any of the given doses.

**Potential mechanisms of IT galanin-mediated antinociception**

The present findings support the assertion that at the spinal level an antinociceptive action is mediated by GalR1 activation. The presence of GalR1 mRNA in DRG and the spinal terminals of primary afferent fibers suggest a modulatory effect upon primary afferent terminal excitability. Previous work has shown that galanin may presynaptically inhibit release of glutamate in CNS (Zini et al., 1993), a finding consistent with the ability of GalR1 to block the opening of voltage sensitive calcium channels (Parsons et al., 1998). In addition, GalR1 mRNA
is present in spinal dorsal horn neurons. It is known that Gal1-29 opens K+ channels leading to hyperpolarization of neurons (Ren et al., 2001); this suggests that postsynaptic action of galanin may be critical in abrogating the downstream events that lead to the spinal sensitization and hyperalgesia. Activation of spinal NMDA receptors is known to trigger a cascade of intracellular events, including activation of enzymes such as phospholipase A2 and nitric oxide synthase that lead to the generation of prostanoids and nitric oxide (NO). These intermediaries contribute to spinal sensitization that underlies the behaviorally defined hyperalgesia arising from peripheral tissue injury and inflammation (see (Yaksh et al., 1999)). Consistent with a direct postsynaptic regulation by GalR1 of the spinal processing initiated by peripheral injury are the observations that galanin antagonizes substance P (SP)-induced neuronal hyperexcitability (Xu et al., 1990), and that IT Gal1-29 blocks spinal SP-evoked hyperalgesia and PGE2 release (Hua et al., 2002). These dual actions on neuronal excitability of afferent and secondary order neurons resembles the motif that has been described for several other agonists with analgesic properties including the µ/δ opioids and α2 adrenergics acting at G protein-coupled receptors (Yaksh et al., 1999). Accordingly, the functional outcome is the suppression of the formalin evoked flinching response by intrathecal galanin.

Synergistic actions of galanin

Previous work has shown that activation of spinal opiate receptors or antagonism of NMDA receptors will attenuate spinal nociceptive processing and prevent development of a facilitated state by reducing injury-induced afferent input and diminishing the downstream cascade (Yaksh et al., 1999). Similar effects were seen in the present study. We sought to define the nature of the interaction between these receptors and the GalR1. The antinociceptive effects
of Gal1-29 are of intermediate potency between those of morphine and AP-5 in the formalin model. Accordingly, we undertook a fixed ratio isobolographic analysis. Considering the interaction between Gal1-29 and morphine or between Gal1-29 and AP-5, a significant synergistic effect was observed between both classes of compounds (Figures 5 and 6). This is likely to be the consequence of different sites of actions on a common cascade of nociceptive processing. GalR1 activation at presynaptic sites may speculatively reduce glutamate and SP release in the spinal cord, and at postsynaptic sites it may counteract the NMDA receptor mediated Ca\(^{++}\) dependent NO and prostanoid formation, which are thought to form the basis of NMDA-mediated nociception (Yaksh et al., 1999). Any of these effects, alone or in combination, correspond with the proposed mechanisms by which spinal opiate receptor activation or antagonism of the NMDA receptors are thought to alter nociceptive processing.

We wish to emphasize that synergistic action of galanin on spinal morphine and AP-5 is of potential clinical significance. Chronic treatment with opioid analgesics such as systemic or spinal morphine leads to tolerance and dependence, effects that limit their therapeutic efficacy. There are clinical studies showing that spinal delivery of an NMDA antagonist can diminish a major component of a post nerve injury pain state (Kristensen et al., 1992), the adverse cognitive effect of these agents are still of major concern (Max et al., 1995). It should be appreciated that low doses of the two drugs administrated simultaneously will produce enhanced analgesic effects with reduced adverse side effects. The potent antinociceptive synergy with no sign of additional side effects suggests GalR1 as a potential target for pain therapy.
In conclusion, while these data do not conclusively exclude a possible role of other GalRs, they do strongly support the hypothesis that spinal GalR1 mediates the inhibitory action of galanin on spinal nociceptive processing, and that this antinociceptive effect is synergistic with that of both opiate receptor activation and NMDA receptor antagonism. Further definition of these actions will require development of more selective agonists and antagonists for GalR1 receptors.
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Footnotes


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Legends for Figures

**Fig. 1** Antinociception of IT Rat Galanin (Gal$_{1-29}$)

(A) Time course over 60-min of flinch responses following formalin paw injection in rats with IT injection of Gal$_{1-29}$ (30nmol, N=7) or saline (10µl, N=9). Each point represents the number of flinches per min. (B) Histograms representing the dose-response effects of Gal$_{1-29}$ (3-30 nmol) on formalin test phase 1 and phase 2 (A & B) flinching behavior. The bars represent the total number of flinches in each phase: Ph1 (1-9 min), Ph2 (10-60 min), Ph2A (10-40 min) and Ph2B (41-60 min). The data are expressed as mean ± s.e.m. of 4-10 rats per group. *p < 0.05 vs. the saline group, one-way ANOVA followed by Dunnett’s tests.

**Fig. 2** Antinociception of IT Human Galanin (Gal$_{1-30}$)

(A) Time course over 60-min of flinch responses following formalin paw injection in rats with the IT injection of Gal$_{1-30}$ (30nmol, N=5) or saline (10µl, N=9). Each point represents the number of flinches per min. (B) Dose-response effects of Gal$_{1-30}$ (3-30 nmol) on formalin test phase 1 and 2 (Ph1/2) flinching behavior. The bars represent the total number of flinches in each phase. N=3-10. *p < 0.05 vs. the saline group, one-way ANOVA followed by Dunnett’s tests.

**Fig. 3** Summary of the effects of IT galanin and the analogues in the formalin test

Histograms representing the effect of all agents given at a dose of 30 nmol (IT) on formalin paw injection induced flinching response in the Phase 1 and 2 (Ph1/2). N=4-13. *p < 0.05 vs. the saline group, one-way ANOVA followed by Dunnett’s tests.

**Fig. 4** Dose-response curves of IT morphine, AP-5 and Gal$_{1-29}$

Dose-response curves of antinociceptive effects of IT morphine (N=22), AP-5 (N=16) and Gal$_{1-29}$ (N=17) on formalin paw injection-induced flinches (2$^{nd}$ phase). The effects are expressed as % control group (saline). The total flinches of the phase 2 of the control group are 1027±87 (N=18). See Table 1 for the ED$_{50}$ values.
Fig. 5 Co-administration of Gal1-29 and morphine
(A) The graph represents the flinching response to formalin paw injection in the rats that had a half ED$_{50}$ dose of either Gal1-29 (Gal, 10 nmol) or morphine (Mor, 2 nmol) alone, or the rats that had a combination of both drugs at their half ED$_{50}$ doses (Gal+Mor). N=5-7. (B) Isobolographic plot for the interaction of the antinociceptive effect of IT Gal1-29 and morphine on formalin phase 2 flinches. The ED$_{50}$ values for the single agents are plotted on the X and Y axes respectively. The line connecting these two points is the theoretical additive line, and the point on this line is the theoretical additive point (and S.E.) calculated from the ED$_{50}$ values and their variance. The experimental point (and S.E.) for the combination fell below the theoretical additive point ($p < 0.05$, $t$-test).

Fig. 6 Co-administration of Gal1-29 and AP-5
(A) The graph represents the flinching response to formalin paw injection in the rats that had a half ED$_{50}$ dose of either Gal1-29 (Gal, 10 nmol) or AP-5 (26 nmol) alone, or the rats that had a combination of both drugs at their half ED$_{50}$ doses (Gal+AP-5). N=5-6. (B) Isobolographic plot for the interaction of the antinociceptive effect of IT Gal1-29 and AP-5 on formalin phase 2 flinches. The ED$_{50}$ values for the single agents are plotted on the X and Y axes respectively. The line connecting these two points is the theoretical additive line, and the point on this line is the theoretical additive point (and S.E.) calculated from the ED$_{50}$ values and their variance. The experimental point (and S.E.) for the combination fell below the theoretical additive point ($p < 0.05$, $t$-test).
Fig. 1
Fig. 2
Fig. 3
Formalin 2nd phase

% control

nmol

○ Morphine
△ AP-5
■ Gal1-29

Fig. 4
**Table 1**

ED$_{50}$ values with 95% Confidence Intervals (C.I) of intrathecal galanin (Gal$_{1-29}$), morphine (Mor) or AP-5 alone, and ED$_{50}$ values of morphine/AP-5 in combination with Gal$_{1-29}$ on flinching response evoked by formalin paw injection

<table>
<thead>
<tr>
<th>Agents</th>
<th>Phase II</th>
<th>Phase IIA</th>
<th>Dose range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal$_{1-29}$</td>
<td>19 (8-47)</td>
<td>11 (5-26)</td>
<td>3 - 30</td>
</tr>
<tr>
<td>Mor</td>
<td>4 (1-11)</td>
<td>2 (1-6)</td>
<td>0.3 - 90</td>
</tr>
<tr>
<td>Mor (Gal$_{1-29}$)@</td>
<td>1 (1-2)</td>
<td>1 (0.7-1.2)</td>
<td>0.45 -1.8</td>
</tr>
<tr>
<td>AP-5</td>
<td>60 (34-105)</td>
<td>54 (37-79)</td>
<td>5.6 -152</td>
</tr>
<tr>
<td>AP-5 (Gal$_{1-29}$)@</td>
<td>15 (10-23)</td>
<td>13 (9-17)</td>
<td>6.5 - 26</td>
</tr>
</tbody>
</table>

@ The combination doses were defined by ratio of the ED$_{50}$ of respective drug given alone.