Receptor subtype-specific pronociceptive and analgesic actions of galanin in the spinal cord: Selective actions via GalR1 and GalR2 receptors

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Galanin is a 29-aa neuropeptide with a complex role in pain processing. Several galanin receptor subtypes are present in dorsal root ganglia and spinal cord with a differential distribution. Here, we describe a generation of a specific galanin R2 (GalR2) agonist, AR-M1896, and its application in studies of a rat neuropathic pain model (Bennett). The results show that in normal rats mechanical and cold allodynia of the hindpaw are induced after intrathecal infusion of low-dose galanin (25 ng per 0.5 μl/h). The same effect is seen with equimolar doses of AR-M1896 or AR-M961, an agonist both at GalR1 and GalR2 receptors. In allodynic Bennett model rats, the mechanical threshold increased dose-dependently after intrathecal injection of a high dose of AR-M961, whereas no effect was observed in the control or AR-M1896 group. No effect of either of the two compounds was observed in nonallodynic Bennett model rats. These data indicate that a low dose of galanin has a nociceptive role at the spinal cord level mediated by GalR2 receptors, whereas the antiallodynic effect of high-dose galanin on neuropathic pain is mediated by the GalR1 receptors. Thus, a selective GalR1 agonist may be used to treat neuropathic pain.

allodynia | Bennett model | dorsal root ganglia | neuropeptide | pain

Galanin is a 29-aa (30 aa in humans) neuropeptide (1). It has a wide distribution in the nervous system and may be involved in a variety of physiological and pathophysiological activities (2), including pain signaling (3–5). Galanin is upregulated in dorsal root ganglion (DRG) neurons in many animal models based on peripheral nerve injury, including complete axotomy (6, 7), complete nerve constriction injury (8–10), as well as partial nerve ligation (9, 10). In agreement, enhanced immunoreactive galanin release was also found in the superficial dorsal horn ipsilateral to sciatic nerve injury (11).

Galanin's role in pain signaling is complex, and early studies revealed inhibitory (12–15) and at low doses excitatory (16–18) effects of galanin, as well as enhanced inhibition after nerve injury (17, 19). It also has been proposed that galanin after nerve injury may contribute to neuropathic pain (9), and recently infusion of low doses of galanin resulted in a decrease in pain threshold (20, 21).

Three galanin receptors have been identified and cloned (22–34), and all of the three subtype mRNAs are expressed within DRGs and spinal cord with different distribution patterns, expression levels, and response to peripheral nerve injury (4, 34–38). All three known receptors belong to the superfamily of G protein-coupled transmembrane receptors, and they use different transduction signaling pathways (see ref. 34). The multitude of receptors may at least in part underlie the diversity and opposing views on the apparent functional roles of galanin in pain processing at the spinal level.

A problem in the field has been the lack of specific and efficient drugs affecting galaninergic mechanisms. However, Bartfai and colleagues (39) have developed a series of chimeric peptide analogues with galanin receptor antagonistic activity. Also, two small, nonpeptide galanin R1 receptor (GalR1-R) antagonists have been found by random screening (40, 41). Schmidt *et al.* (42) have synthesized peptide analogue AR-M961, [Sar(1), D-Ala^{12}]Gal(1–16)-NH_2, which has high affinity and functional activity for both the GalR1-R and GalR2-R. In the present study, we describe development of an agonist, AR-M1896 [Gal(2–11)Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-NH_2], with selective GalR2 agonist activity. Moreover, we compare the effects of AR-M961 and AR-M1896 on pain behavior after acute and chronic intrathecal (i.t.) injection under different conditions to explore the receptor mechanisms underlying the varying roles of galanin in pain processing at the spinal level.

Materials and Methods

Characterization of a Galanin Receptor Ligand. Receptor binding and functional assays were performed exactly as described (43). Briefly, receptor binding assays measured the potency of test ^{125}I-human (h) galanin (≈0.04 nM) from membranes of HEK293S cells expressing rat (r) GalR2-Rs and from Bowes melanoma cells expressing hGalR1-Rs. These assays were performed in 50 mM Tris/3 mM $MgCl_2$/2.5 mg/ml BSA/3.75 μM β-endorphin, pH 7.4, and were terminated at equilibrium by filtration. The functional assay for hGalR1 measured the potency of test compounds in stimulating guanosine 5′-[γ-thio]triphosphate binding to the Bowes melanoma cell membranes in 50 mM Hepes/20 mM NaOH, pH 7.4/5 mM $MgCl_2$/100 mM NaCl/1 mM EDTA/0.1% BSA/15 μM GDP. The functional assay for rGalR2 measured the potency of test compounds in stimulating the mobilization of intracellular calcium in HEK293S-rGalR2 cells loaded with Fluo-3. These experiments were performed by using a Fluorescence Imaging Plate Reader (Molecular Devices) in Hanks' buffer supplemented with 20 mM Hepes plus 0.1% BSA, pH 7.4.

Effects of Galanin Agonists on Pain Behavior. *Animals.* Adult male Sprague–Dawley rats (240–260 g) (B & K Universal, Sollentuna, Sweden) were used. The rats were housed in cages at room temperature (20–25°C) under a 12/12-h light/dark cycle with free access to food and water. The experiments were conducted according to the Ethical Guidelines for Investigation of Experimental Pain in Conscious Animals (44) and were approved by the local ethics committee for animal research.

Unilateral sciatic nerve injury. Unilateral sciatic nerve injury was produced under deep anesthesia with i.p injection of sodium pentobarbital (Mebumal, 60 mg/kg), as described by Bennett

Abbreviations: GalR1-R and GalR2-R, galanin R1 and R2 receptors; i.t., intrathecal; DRG, dorsal root ganglia; h, human; r, rat.

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Table 1. Characterization of binding and functional activity of galanin analogs

Analog	Sequence	Binding IC_{50}, nM		hGalR1/ rGalR2	FLIPR hGalR2 EC_{50}, nM	GTP[γS] hGalR1 EC_{50}, nM
		rGalR2	hGalR1			
hGal	Human galanin	1.00	0.23	0.23	6.25	10.7
AR-M1717	Gal(1-11)-NH_2	1.65	1.10	0.66	6.29	4400
AR-M1896	Gal(2-11)-NH_2	1.76	879	500	9.32	—*
AR-M961	[Sar[1], D-Ala[12]]gal(1-16)-NH_2	1.74	0.403	0.23	7.12	164

FLIPR, fluorescence imaging plate reader; GTP[γS], guanosine 5′-[γ-thio]triphosphate.
*Not tested because the binding assay showed IC_{50} is 879 nM. All analogs tested showed that binding in the high nanomolar range is accompanied by an almost complete loss of GalR1 activation.

and Xie (45). The common sciatic nerve was exposed and freed for about 10 mm at mid-thigh level. Four ligatures (Ethicon, 4.0 plain gut) were tied loosely around the nerve with about 1-mm spacing, and the incision was closed.

Intrathecal catheterization and injection. A chronic i.t. catheter (PE 10, o.d. 0.61 mm) was implanted between the L5 and L6 vertebrae under anesthesia (as above) (46) in naïve rats or rats 7 days after loose sciatic nerve ligation, with its tip at the lumbar enlargement. Intrathecal injection and behavioral measurement of the hindpaw were performed 7 days later. The proper location of the catheter was tested 24 h before the pharmacological experiments by assessing sensory and motor blockade after i.t. injection of 7 μl of lidocaine (50 mg/ml, AstraZeneca R & D, Montreal).

Implantation of the miniosmotic pumps. Twenty-nine normal rats with implanted i.t. catheter (5 days earlier) received low-dose galanin or one of the agonists continuously for 14 days. The miniosmotic pumps (Alzet, model 2002, 0.5 μl/h) filled with vehicle [1 mg of rat serum albumin (Sigma), 1 mg of ampicillin (Pentrexyl; Bristol-Myers Squibb] in 1 ml of sterile 0.9% NaCl solution, galanin (50 μg/ml vehicle, Bachem) or the agonists (AR-M1896, 19.8 μg/ml vehicle and AR-M961, 28.4 μg/ml vehicle) at equimolar concentrations (15.8 μM) were implanted s.c. and connected to the i.t. catheter, and the solutions were delivered at a constant velocity (25 ng of galanin/h, 9.88 ng of AR-M1896/h, 14.2 ng of AR-M961/h; corresponding to 7.9 pmol/h). All peptides were dissolved in distilled water and diluted with sterile saline. They were coded and stored in aliquots at −20°C until use. The mechanical threshold and cold score were measured 0, 2, 4, 6, 8, 10, and 13 days after the pump implantation. The catheter location was examined with lidocaine before the animals were killed. The values from the side with the most complete sensory lidocaine blockade were used.

Behavioral testing. All of the behavioral testing was performed during daytime (9.00–18.00). Rats were placed in transparent plastic domes (8 × 8 × 18 cm) on a metal mesh floor with a hole size of 3 × 3 mm. The measurements were performed after 15 min of adaptation.

Measurement of mechanical threshold. A series of von Frey filaments (0.5, 0.88, 1.28, 2.7, 5.1, 7.5, 8.8, 13.5, and 23 g) (23 g was taken as cut-off because stronger filaments could lift the hindlimb of the rat) were applied in ascending manner from below at the center of the plantar surface of the hindpaw ipsilateral to the nerve injury in Bennett model rats and bilaterally on the hindpaws of the pump-implanted normal rats. Each filament was delivered three times with ≈2-s intervals. The lowest force at which each of the three applications of the filament elicited a brisk hindpaw withdrawal was taken as the mechanical response threshold.

Assessment of cold score. Brisk foot withdrawal in response to acetone application was measured based on the method described by Choi *et al.* (47). The acetone bubble was gently brought in touch with the plantar surface around the center, and the acetone quickly spread over the central part of the plantar surface of the foot. Applications were made five times (once every 5 min) to each paw. The score for each application was recorded as follows: foot withdrawal was scored as positive (1) and lack of withdrawal as negative (0). The total score (0 to 5) was taken as index for cold sensitivity of the foot.

Experimental design. All of the behavioral measurements for the data shown in the present study were conducted in a blind manner. Twenty-nine normal rats were randomly divided into four groups (seven or eight rats in each). A low dose of galanin agonist was constantly i.t. delivered by a miniosmotic pump. Infusion of vehicle and low-dose galanin was performed as negative and positive controls, respectively.

In Bennett model rats, the measurement of the mechanical threshold started 14 days after the nerve injury. The basal threshold was usually between 2.85 and 23 g. The rats were chosen and divided into two groups according to the threshold, nonallodynic (≥23 g) and allodynic (≤5.1 g) rats. In each group, the effect of a high i.t. dose of each agonist was recorded. Intrathecal injection of the same volume (10 μl) of saline was used as control. In the first experiment, a very high dose (20 μg) of galanin agonist or vehicle was administered i.t. in six allodynic rats and three nonallodynic rats, and the effect was tested blindly. We then used lower doses (1 μg and 9 μg) of AR-M961 to examine the dose dependence of its antiallodynic effect in allodynic model rats in a nonblind manner (data not shown). To further confirm the effects of the two agonists in allodynic and nonallodynic rats, a second series of blind experiments was performed in an alternate injection manner (seven allodynic and five nonallodynic rats) by using a regime with increasing doses (0.1, 1, and 10 μg).

Statistics. The results were presented as median ± median-derived absolute deviation. Friedman ANOVA (one-way repeated measures ANOVA on ranks) was used to analyze the data in each group with the time course. The Kruskal–Wallis ANOVA test (one-way ANOVA on ranks) was used for the comparison of data among groups at the same time point. A P value less than 0.05 was chosen as the significant level.

Results

Characteristics of AR-M1896. Structure–activity studies of galanin revealed that the C-terminally truncated Gal(1–11)-NH_2 is the shortest nonselective high-affinity ligand for GalR1-R and GalR2-R (Table 1). Although this analog binds with high affinity to GalR1, its functional activity is extremely low with an EC_{50} value of only 4.4 μM. Additional removal of the glycine residue in position 1 resulted in Gal(2–11)-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-NH_2 (AR-M1896) with almost unchanged GalR2 affinity and functional activity, and 500-fold selectivity for GalR2-Rs over GalR1-Rs. This compound represents a truly GalR2-selective galanin analog and, therefore, could be used as a pharmacological tool to differentiate between these two receptors.

Allodynic Effects of Low Dose of Galanin Agonists in Normal Rats. The mechanical threshold was dramatically reduced (Fig. 1*A*), and the cold sensitivity to acetone of the hindpaw increased signif-

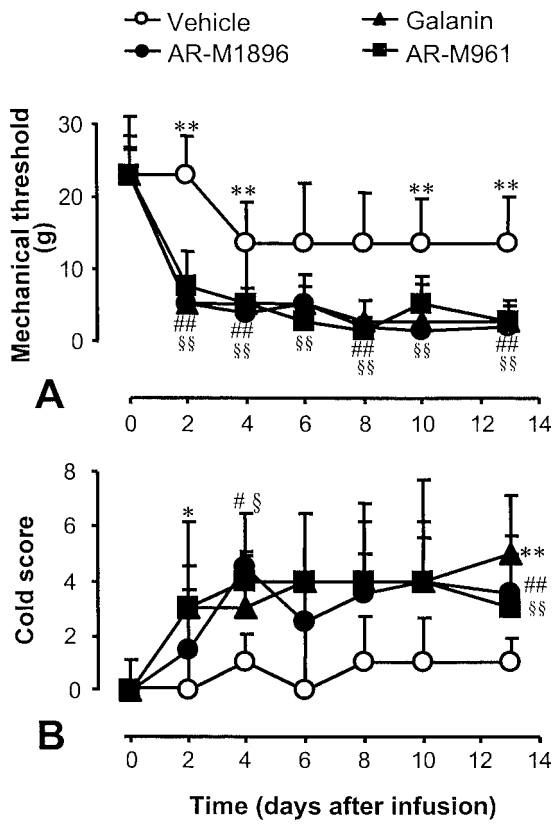

Fig. 1. Effects of i.t. infusion of low-dose galanin agonists on the mechanical (*A*) and cold (*B*) sensitivity of the hindpaw in normal rats. Both mechanical and cold sensitivity of the hindpaw increased significantly after i.t. infusion of either agonist (Friedman ANOVA, $P < 0.05$), whereas infusion of vehicle did not induce any significant change of von Frey hair threshold or cold score (Friedman ANOVA, $P > 0.05$). At 2 days after i.t. infusion, the von Frey hair threshold was lower in the three treatment groups than in the control group (Kruskal–Wallis ANOVA, $P < 0.01$). The cold score increased 2 days after infusion of galanin ($P < 0.05$) and 4 days after infusion of AR-M1896 or AR-M961 ($P < 0.05$). * and ** (galanin group), # and ## (AR-M1896 group), and § and §§ (AR-M961 group) indicate $P < 0.05$ and $P < 0.01$, respectively, compared with vehicle group at the same time point (one-way ANOVA on ranks).

icantly (Fig. 1*B*) after i.t. infusion of galanin (25 ng per 0.5 μl/h), AR-M1896 (9.88 ng per 0.5 μl/h), or AR-M961 (14.2 ng per 0.5 μl/h) (Friedman ANOVA, $P < 0.01$), whereas infusion of vehicle did not induce any significant change, neither with regard to von Frey hair threshold nor to cold score ($P > 0.05$) (Fig. 1). The von Frey hair threshold was lower in the three treatment groups than in the control group 2 days after i.t. infusion (Kruskal–Wallis ANOVA, $P < 0.01$) (Fig. 1*A*). The cold score increased after 2 days of galanin infusion ($P < 0.05$) and after 4 days of infusion of AR-M1896 or AR-M961 ($P < 0.05$) (Fig. 1*B*).

Antiallodynic Effect of AR-M961 in the Bennett Neuropathic Pain Model. In allodynic rats, the mechanical threshold dose-dependently increased after i.t. injection of AR-M961 (Friedman ANOVA, $P < 0.001$). The threshold was significantly higher than in the control group 7.5 and 15 min after injection of 1 μg (Kruskal–Wallis ANOVA, $P < 0.05$ and $P < 0.01$ respectively), 7.5, 15, and 30 min after injection of 10 μg ($P < 0.01$ for all), and 15 and 30 min after injection of 20 μg ($P < 0.01$ and $P < 0.05$, respectively) of AR-M961. No change was observed in control and AR-M1896-treated groups (Friedman ANOVA, $P > 0.05$) (Fig. 2*A*).

In nonallodynic rats, neither galanin agonists nor vehicle induced any significant change of the mechanical threshold of the hindpaw ($P > 0.05$) (Fig. 2*B*).

Discussion

Here, we present a galanin analogue, AR-M1896 [Gal(2–11)-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-NH_2], with high selectivity for the GalR2-R. In combination with galanin and the previously published analogue AR-M961, [Sar(1), D-Ala[12]]Gal(1–16)-NH_2, which binds with about the same affinity to both GalR1-R and GalR2-R, we can now attempt to differentiate behaviors specific for these two receptors. These two compounds and galanin were given i.t. to normal rats and rats with loose, chronic nerve constriction, a neuropathic rat pain model developed by Bennett and Xie (45). Our results may at least in part explain the variety of behavioral and electrophysiological responses reported in the literature, as well as some of the controversies around the role of galanin in pain signaling, and suggest that presence, differential distribution patterns, and expression levels of the three galanin receptors GalR1, GalR2, and GalR3 in DRGs and/or spinal cord (24, 33–36, 38, 48, 49) are important factors. Thus, the GalR1-R seems responsible for inhibition after nerve injury, whereas the GalR2-R may mediate the excitatory effect of low-dose galanin in normal rats.

In several studies, exogenous galanin has been applied onto the spinal cord. Thus, a single i.t. injection of a low dose of galanin (0.1 and 1 nmol) increased reflex excitability in normal rats (18) and decerebrate, spinalized, unanesthetized rats (15, 16). Furthermore, galanin dose-dependently enhanced Aδ- and C fiber-evoked responses, postdischarge, and wind up, demonstrating a pronociceptive role (21). Moreover, chronic intrathecal delivery of a low dose (25 ng/h, 14 days) of exogenous galanin to nerve-intact adult rats, assumed to mimic the situation with increased galanin release after nerve injury, induced persistent mechanical hypersensitivity (20). Finally, Kerr *et al.* (20) found that after full sciatic nerve transection or partial nerve injury, spontaneous and evoked neuropathic pain behaviors are largely eliminated or severely compromised in galanin null mutant mice. These and other (9) data suggest that the up-regulation of galanin is associated with the development of neuropathic pain after peripheral nerve injury.

Here, we used the same paradigm as Kerr *et al.* (20) as a positive control and found a dramatic and persistent decrease of mechanical threshold of the hindpaw after i.t. infusion of a low dose of galanin, thus fully confirming their report. A moderate thermal hypersensitivity of the hindpaw was observed in our experiment, but not by Kerr *et al.* (20). Furthermore, i.t. infusion of equimolar doses of the galanin receptor agonists AR-M1896 and AR-M961, which have the same affinity and functional activity at the GalR2-R, induced the same effects as galanin on the mechanical and thermal sensitivity, indicating that the GalR2-Rs mediate the excitatory action of galanin in the spinal cord.

There is evidence that high levels/doses of galanin have antinociceptive effects. Thus, i.t. administration of galanin at 1 μg or more inhibits the nocifensive reflex in spinalized (16, 17) and normal (13, 50) rats, in contrast to the facilitatory effect of low doses described above. Moreover, very high doses (≥10 μg) of exogenous galanin alleviate neuropathic pain behaviors after peripheral nerve injury (51, 52). In our previous study (53), we examined the effect of exogenous and endogenous galanin on pain behavior in nonallodynic and allodynic Bennett model rats, two groups in which galanin levels in DRGs have been reported to be different (43% of neuron profiles expressed galanin-like immunoreactivity in nonallodynic rats versus 23% in the allodynic group) (10). We found that i.t. galanin induces an antiallodynic effect in allodynic rats that is in agreement with other reports (51, 52). Moreover, in nonallodynic Bennett model

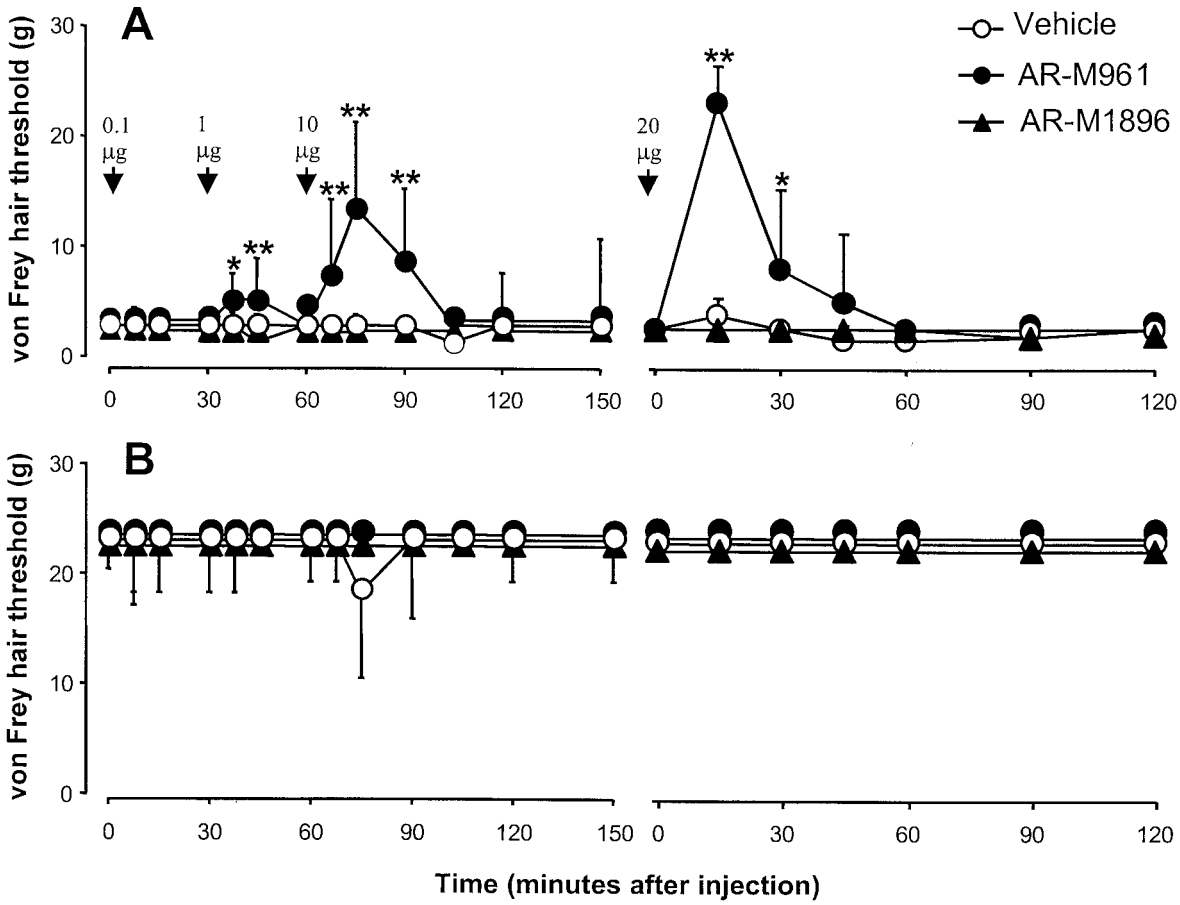

Fig. 2. Effects of galanin agonists (AR-M1896 and AR-M961) on the mechanical threshold in allodynic (*A*) and nonallodynic (*B*) Bennett model rats. In allodynic rats, the mechanical threshold dose-dependently increased after i.t. injection of AR-M961 (repeated measures ANOVA on ranks, $P < 0.001$). The threshold was significantly higher than control group at 7.5 and 15 min after injection of 1 μg (one-way ANOVA on ranks, $P < 0.05$ and $P < 0.01$, respectively) at 7.5, 15, and 30 min after injection of 10 μg ($P < 0.01$ for all), and at 15 and 30 min after injection of 20 μg ($P < 0.01$ and $P < 0.05$, respectively) of AR-M961. No change was observed in control and AR-M1896 groups (repeated measures ANOVA on ranks, $P > 0.05$). In nonallodynic rats, neither galanin agonists nor vehicle induced any significant change of the mechanical threshold of the hindpaw (repeated measures ANOVA on ranks, $P > 0.05$). * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, compared with control at the same time point (one-way ANOVA on ranks).

rats, the putative galanin receptor antagonist M35 (see ref. 39), when given i.t., dose-dependently induced a long-lasting allodynic state, indicating that high-level endogenous galanin exerts a tonic inhibition of pain processing in the spinal cord after nerve injury (53). In the present study, i.t. AR-M961 increased the von Frey hair threshold dose-dependently in allodynic Bennett model rats, whereas no change of the threshold was observed with the selective GalR2 agonist AR-M1896 or vehicle. Thus, the antiallodynic effect exerted by galanin in nerve-injured rats seems to be mediated by GalR1-Rs.

We also studied the effect of the two galanin agonists on the mechanical threshold in nonallodynic Bennett model rats. Neither of them induced any change of the threshold, even though both of them can activate GalR2-Rs, which mediate the excitatory effects of galanin in nerve-intact rats.

A high density of galanin binding sites has been shown with receptor autoradiography in laminae I and II of the rat spinal cord (54–56). With *in situ* hybridization, GalR1-R mRNA, under normal circumstances, can be observed in about 20% of all, mostly calcitonin gene-related peptide-positive neuron profiles in rat L4 and L5 DRGs (35), mainly constituting large- and medium-sized neurons (38). About 25% of all DRG neuron profiles are GalR2-R mRNA-positive, mainly of the small type, and 80% of them colocalized with calcitonin gene-related peptide mRNA and about 20% with GalR1-R mRNA (36, 38). So far, GalR3-R expression has been detected only with reverse transcriptase-PCR and solution hybridization/RNase protection assay, both in DRGs and spinal cord, but the levels are lower than those of GalR1 and GalR2 receptor mRNAs (49).

In the rat spinal cord, many intrinsic neurons in laminae I and II express GalR1-R mRNA, with some neurons in laminae III–V, whereas only a few neurons express GalR2 mRNA in the dorsal horn (24, 38, 48). Thus, under normal conditions the main galanin receptor subtypes presumably associated with pain transmission at the spinal level are GalR1-Rs in intrinsic dorsal horn neurons and GalR2-Rs in DRG neurons, whereby it is not known to what extent the latter receptor indeed is transported centrally in sensory afferents to the dorsal horn. However, a parallel electrophysiological study on the effect of AR-M1896 and AR-M961 on locus coeruleus neurons, which contain both GalR1 and R2 mRNA (38), indicated that the GalR1-R is postsynaptic and the GalR2-R is presynaptic (X. Ma, Y.-G. Tong, R.S., W.B., K.P., L.H., C.P., C.G., T.H., and Z.-Q.D. Xu, unpublished results). It may thus be speculated that i.t. injection of low doses of galanin may bind to GalR2-Rs on dorsal horn primary afferent endings to activate phospholipase C pathways by Gq to increase the Ca^{2+} concentrations (27, 57), stimulating nociceptive transmission by modulating release of coexisting neurotransmitters, e.g., glutamate, substance P, and/or calcitonin gene-related peptide. The inhibitory role, mainly seen after

nerve injury and usually induced by high-dose galanin, perhaps mainly results from inhibition of cAMP production mediated by G_i-coupled GalR1-Rs (24, 57) on intrinsic dorsal horn neurons.

In situ hybridization shows that the number of GalR1-R and GalR2-R mRNA-positive neurons in rat DRGs is reduced after axotomy (35, 36). After sciatic nerve transection, Kar and Quirion (55) reported a decrease in galanin binding sites in the dorsal horn, perhaps at least in part reflecting the decrease in GalR1-R and/or GalR2-R synthesis in DRGs. On the other hand, axotomy had no effect on GalR1-R mRNA in dorsal horn neurons (37). The lesion-induced down-regulation of GalR1-R and especially GalR2-R in DRG neurons may thus lead to a decrease in GalR2-Rs on primary afferents and shift the balance in the dorsal horn toward a stronger influence of the postsynaptic inhibitory GalR1-R. This would be in agreement with the results that the GalR2-R agonist AR-M1896 failed, even at high dose, to induce any excitatory effect in nonallodynic Bennett model rats, as it did at low dose in normal rats. It also agrees with the enhanced inhibitory role of endogenous galanin after peripheral nerve injury (17, 19) and with the inhibitory effect of i.t. galanin in some rat neuropathic pain models (51–53). Accordingly, the present results on the allodynic Bennett model rats are compatible with the view that it is the GalR1-R, and not the GalR2-R, that mediates the inhibitory effect of galanin on pain behavior. With regard to humans, it is known that human DRGs express galanin (37), but expression of receptor subtypes in DRGs and spinal cord has not been explored. Should the situation be similar in humans and rats, possibilities to use selective GalR1-R agonists for treatment of neuropathic pain could open up.

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