Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 3334–3337, April 1992
Neurobiology

Galanin-mediated control of pain: Enhanced role after nerve injury

ZSUZSANNA WIESENFELD-HALLIN*, XIAO-JUN XU*, ÜLO LANGEL†, KATARINA BEDECS†, TOMAS HÖKFELT‡, AND TAMAS BARTFAI†

*Department of Clinical Physiology, Section of Clinical Neurophysiology, Karolinska Institute, Huddinge University Hospital, Huddinge, †Department of Biochemistry, University of Stockholm, Stockholm, and ‡Department of Histology and Neurobiology, Karolinska Institute, Stockholm, Sweden

Contributed by Tomas Hökfelt, January 6, 1992

ABSTRACT **The endogenous inhibitory role of the neuropeptide galanin in pain transmission and spinal cord excitability was demonstrated by the use of a high-affinity galanin receptor antagonist, M-35 [galanin-(1–13)-bradykinin-(2–9)-amide]. M-35, which displaced ^{125}I-labeled galanin from membranes of rat dorsal spinal cord with an IC_{50} of 0.3 nM, dose-dependently antagonized the effect of intrathecal galanin on the flexor reflex. M-35 potentiated the facilitation of the flexor reflex by conditioning stimulation of cutaneous unmyelinated afferents in rats with intact nerves and the potentiating effect of M-35 on the conditioning-stimulation-induced reflex facilitation of the cutaneous unmyelinated afferents was strongly enhanced after axotomy. These results demonstrate that endogenous galanin plays a tonic inhibitory role in the mediation of spinal cord excitability, and it is particularly noteworthy that this function of galanin is remarkably enhanced after peripheral nerve section.**

The neuropeptide galanin (1) consists of 29 amino acids, has been shown to occur in a relatively small population of dorsal root ganglion cells and spinal cord interneurons (2–4), and may be involved in sensory modulation. Although some reports indicated that intrathecal (i.t.) galanin has a hyperalgesic effect and i.t. galanin antibody produces analgesia (5), overwhelming evidence suggests that galanin primarily has an inhibitory effect in sensory transmission at the spinal level. Thus, i.t. galanin blocks the facilitatory effect of the flexor reflex produced by the conditioning stimulation (CS) of unmyelinated afferents and by i.t. applied excitatory neuropeptides, such as substance P and calcitonin gene-related peptide (6–8). Galanin also selectively depresses spinal nociceptive reflexes in adult and newborn rats with no or very limited effect on the monosynaptic reflex (6, 9, 10). Galanin (i.t.) has been reported to cause analgesia without motor deficits (11) and iontophresis of galanin hyperpolarizes dorsal horn neurons (12). Galanin also inhibits the peripheral consequences of antidromic activation of cutaneous unmyelinated (C) afferents, such as the plasma extravasation, mediated by the release of substance P (13, 14).

Peripheral axotomy has dramatic effects on peptidergic sensory afferents. Substance P and calcitonin gene-related peptide are downregulated whereas vasoactive intestinal peptide and galanin are upregulated (15–20). We have recently demonstrated that the vasoactive intestinal peptide level is increased in the same sensory neurons that previously produced substance P (21) and that vasoactive intestinal peptide took over the role of tachykinins as excitatory mediators of nociceptive input (22). We have also shown that the depressive effect of galanin on the flexor reflex is enhanced after nerve section (23) and it antagonized the excitatory effect of vasoactive intestinal peptide, with which it coexists in axotomized sensory neurons (7). Based on these findings, we have hypothesized that one role for upregulated galanin in sensory neurons may be to depress axotomy-induced hyperexcitability in the somatosensory system, thus reducing neuropathic pain (7, 20).

The development of a series of chimeric peptides (24) that bind with high affinity to galanin binding sites in the central nervous system and represent galanin receptor antagonists (24, 25) has allowed us to analyze possible effects of endogenous galanin in the spinal sensory system. In the present study, the effect of M-35 [galanin-(1–13)-bradykinin-(2–9)-amide] on flexor reflex excitability was examined after i.t. injection in rats with intact and sectioned sciatic nerves, and its ability to displace porcine galanin monoiodinated with ^{125}I on Tyr-26 from receptors in membranes of rat lumbar dorsal spinal cord (LDSC) was studied. Furthermore, M-35 consists of the N-terminal fragment of galanin and the C-terminal fragment of bradykinin, but it is unclear to what extent M-35 interacts with bradykinin receptors. Since bradykinin receptors have been found on sensory neurons (26), we have performed additional experiments to examine the effect of i.t. bradykinin and the bradykinin antagonist [D-Arg^0,Hyp^{2,3},D-Phe^7]bradykinin (where Hyp is hydroxyproline) on the facilitation of the flexor reflex induced by C-fiber CS to exclude the possibility that effects seen with M-35 are due to activation or blockade of bradykinin receptors.

MATERIALS AND METHODS

Physiological Experiments. Physiological experiments were carried out on female Sprague–Dawley rats weighing 200–250 g (Alab, Stockholm). The magnitude of the polysynaptic hamstring flexor reflex in response to activation of high-threshold afferents was examined in decerebrate spinal-cord-sectioned unanesthetized rats. In some animals, the sciatic nerves were intact. In the remaining rats, the tibial and peroneal branches of the sciatic nerves were unilaterally ligated and sectioned distal to the ligation at the level of the popliteal fossa under methohexital (Brietal, Eli Lilly, 70 mg/kg, i.p.) anesthesia 10–18 days prior to the acute physiological experiments. In axotomized rats, the sural branch of the sciatic nerve was ligated and sectioned as distally as possible so that it could be electrically stimulated proximal to the site of section without activation of the peroneal or tibial nerves.

In acute experiments, the animals were initially briefly anesthetized with methohexital (Brietal, Eli Lilly, 70 mg/kg, i.p.) and a tracheal cannula was inserted. The rats were decerebrated by aspiration of the forebrain and midbrain and then ventilated. The spinal cord was exposed by a laminectomy at mid-thoracic level and sectioned at Th8-9. An i.t. catheter (PE 10) was implanted caudally to the transection with its tip on the lumbar spinal cord (L4-5). The flexor reflex was elicited by supramaximal test stimuli to the sural nerve

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: i.t., intrathecal; CS, condition stimulation; M-35, galanin-(1–13)-bradykinin-(2–9)-amide; LDSC, lumbar dorsal spinal cord.

or its innervation area in the left foot with electric shocks (0.5 ms, 10 mA, 1 shock per min) of sufficient strength to activate C-afferents (27). The flexor reflex was recorded as electromyographic activity through stainless steel needle electrodes inserted into the ipsilateral posterior biceps femoris/semitendinosus muscles. The number of action potentials elicited during the reflex was integrated over 2 s. During the experiments the heart rate and rectal temperature were monitored. The proper location of the i.t. catheter was confirmed by laminectomy after the experiments.

Binding Experiments. The binding studies were carried out on male adult Sprague–Dawley rats. The membrane fraction from the rat LDSC used in binding studies was prepared as described (28). Briefly, the LDSC was rapidly dissected from decapitated rats, homogenized, and centrifuged at 1000 × *g* for 10 min. The supernatant was further centrifuged at 10,000 × *g* for 45 min, and the resulting pellet was resuspended in 5 mM Hepes-buffered Krebs–Ringer solution [137 mM NaCl/2.68 mM KCl/1.8 mM $CaCl_2$/glucose (1 g/liter)], supplemented with bacitracin (1 mg/ml) and 0.05% bovine serum albumin at pH 7.4.

Synthetic porcine galanin was iodinated by chloramine-T method to yield porcine galanin monoiodinated with ^{125}I on Tyr-26 (specific activity, 1800–2000 Ci/mmol; 1 Ci = 37 GBq) for equilibrium ligand binding studies that used the filtration technique as described (28), except that bacitracin (1 mg/ml) was added in the assay buffer.

Displacement experiments were carried out in a bacitracin-containing (1 mg/ml) 5 mM Hepes-buffered Ringer solution (pH 7.4) containing 0.05% bovine serum albumin in the presence of 0.1–0.2 nM ^{125}I-labeled galanin, the membrane preparation, and increasing concentrations (1 pM to 1 μM) of unlabeled porcine galanin or other galanin receptor ligands. The IC_{50} values of the displacing ligands were calculated by fitting the experimental data on a Macintosh SE by means of a nonlinear least squares method using the program KaleidaGraph. The IC_{50} values were determined in three experiments in duplicates.

Peptides. Porcine galanin used in binding studies was synthesized according to the technique described (24), as was the M-35 used in the binding and physiological experiments. Porcine galanin, bradykinin, and the bradykinin antagonist [D-Arg^0,$Hyp^{2,3}$,D-Phe^7]bradykinin (where Hyp is hydroxyproline) used in physiological experiments were obtained from Bachem. They were dissolved in 0.9% NaCl (saline) and injected i.t. in a volume of 10 μl followed by 10 μl of saline to flush the catheter.

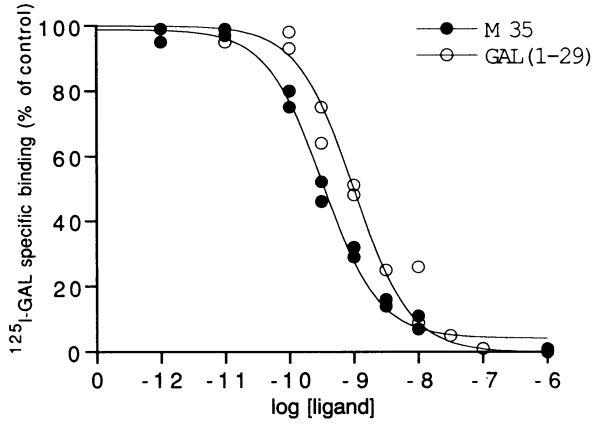

FIG. 1. Displacement of porcine galanin-(1–29) monoiodinated with ^{125}I on Tyr-26 (^{125}I-galanin) (0.1 nM) from membranes of the LDSC by increasing concentrations of porcine galanin-(1–29) and M-35. Specific binding represented 90% of total binding (control). Nonspecific binding was defined as the binding not displaced by 1 μM galanin.

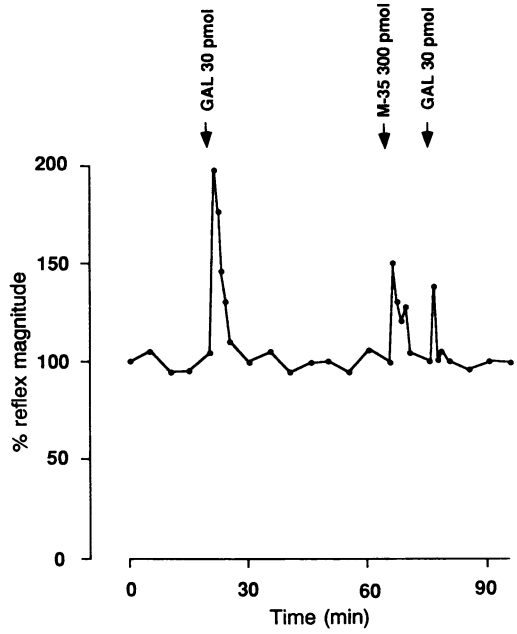

FIG. 2. Illustration of the antagonism by 300 pmol of i.t. M-35 of the facilitation of the flexor reflex induced by 30 pmol of galanin (GAL); 10 μl of saline was used to flush the catheter. The effects of galanin or M-35 were calculated as percent change in reflex magnitude compared to baseline (defined as 100%). M-35 was injected 10 min prior to galanin and antagonized the facilitatory effect of galanin by about 60% in this experiment.

RESULTS

M-35 in a membrane preparation from rat LDSC fully displaced 0.2 nM ^{125}I-labeled galanin in a concentration-dependent manner (Fig. 1). The IC_{50} value for M-35 was 0.3 nM.

In rats with intact sciatic nerves, i.t. galanin at 30 pmol facilitated the flexor reflex (Fig. 2). The maximal increase in reflex magnitude after galanin was 101.0 ± 21.2% lasting 4.6 ± 0.9 min (n = 10). M-35 (i.t.) also dose-dependently facilitated the flexor reflex (Fig. 2). Administered 5–10 min before i.t. galanin, M-35 dose-dependently antagonized the galanin-induced facilitation of the flexor reflex (Fig. 2 and Table 1). No depression of the flexor reflex was observed after i.t. M-35 in the dose range 3–3000 pmol.

CS of cutaneous C-afferents induced a brief increase in spinal cord excitability, demonstrated as a period of facilitation of the flexor reflex after termination of the CS (Fig. 3 and Table 2, see also refs. 6 and 27). We have shown (6–8) that this C-fiber CS-induced central sensitization is blocked by galanin pretreatment of rats with intact and sectioned sciatic nerves. M-35 (i.t.) significantly potentiated the C-fiber CS-induced facilitation of the flexor reflex in rats with intact sciatic nerves (Fig. 3*A* and Table 2). Ten to 15 days after axotomy, this CS facilitated the reflex to the same extent as in rats with intact nerves (Fig. 3*B* and Table 2). In axotomized rats, the potentiation of C-fiber CS-induced reflex facilitation by i.t. M-35 was significantly stronger than that in rats with intact sciatic nerves (Fig. 3*B* and Table 2). No effect of C-fiber CS-induced facilitation was found by i.t. bradykinin or bradykinin antagonist at a dose range of 80 pmol to 8 nmol (data not shown), thus ruling out the possibility that the effect seen with M-35 was due to its interaction with bradykinin receptors in the spinal cord.

Table 1. Antagonistic effect of i.t. M-35 on facilitation of the flexor reflex by 30 pmol of i.t. galanin

M-35	*n*	% antagonism
3 pmol	5	6.6 ± 4.8
30 pmol	6	38.7 ± 8.4*
300 pmol	5	65.0 ± 4.2*
3 nmol	5	76.2 ± 1.6*

Peak facilitatory effect of galanin was 101.0 ± 21.2% over baseline reflex magnitude. The antagonism of galanin by M-35 was calculated as the percent reduction of the peak facilitatory effect of galanin. Data are expressed as the mean ± SEM. The analysis of variance followed by Dunnett's test indicated that M-35 significantly antagonized the galanin-induced reflex facilitation ($F_{3,17,}$= 26.885; $P <$ 0.001). *$P < 0.005$ compared to zero antagonism.

DISCUSSION

M-35, the antagonist introduced in this study, is a member of a family of chimeric-peptide galanin antagonists that bind with high affinity ($K_d < 1$ nM) to neuronal galanin receptors (24). M-15 [galanin-(1–13)-substance P-(5–11)amide], a less-stable analogue of M-35, was shown to antagonize the inhibitory action of exogenous galanin on acetylcholine release in the hippocampus (25) and on insulin release in the pancreas (29). M-15 also antagonized the action of exogenous galanin in the locus coeruleus and spinal cord (25). Here we report that a more-stable analogue, M-35, also has high affinity for galanin receptors in the spinal cord. By using this high-affinity antagonist to galanin, the present study demonstrates a physiological involvement of this peptide in the modulation of spinal sensory processing. Thus, i.t. M-35 exerts a facilitatory effect on flexor reflex excitability and potentiates the reflex facilitation by activation of C-fibers in rats with intact and sectioned sciatic nerves. It is unlikely that the facilitatory effect of M-35 on the flexor reflex is due to a partial agonistic property since unlike galanin (6), this compound did not block, but in fact potentiated, CS-induced reflex facilitation. Thus, the central sensitization phenomenon [i.e., the increase of spinal cord excitability after repetitive stimulation of unmyelinated afferents (27)] is significantly potentiated by M-35 in rats with intact nerves and particularly so after axotomy, suggesting that endogenous galanin is released upon intense activation of nociceptors and that its role under these circumstances is inhibitory. The increase in the potentiating effect of M-35 after axotomy may be related to increased galanin release, reflecting the increase of galanin-like immunoreactivity shown (19–21) to occur in primary sensory afferents after such a lesion. Thus, the increase of galanin in axotomized sensory fibers may be a response to the hyperexcitability of injured afferents (30) that may underlie neuropathic pain. In support of this hypothesis, we have found that chronic i.t. infusion of M-35 markedly enhanced autotomy behavior, a sign of neuropathic pain, in rats after sciatic nerve section (unpublished results).

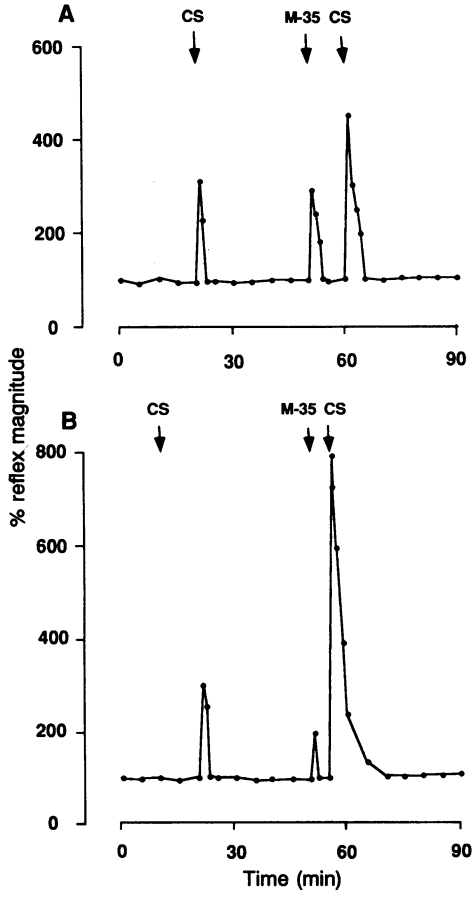

FIG. 3. Illustration of the potentiation of the facilitation of the flexor reflex induced by a CS train (20 shocks at 0.9 Hz) to C-fibers in the sural nerve in a rat with intact sciatic nerves (*A*) and in a rat 12 days after unilateral axotomy (*B*). The CS train was delivered to sural nerve afferents with the same intensity as the test stimuli to evoke the flexor reflex and elicited similar facilitation under both conditions, which was potentiated by M-35 administered 10 (*A*) or 5 (*B*) min prior to the CS. Note that the potentiating effect of M-35 was much stronger in the axotomized rat.

Table 2. Potentiation of the CS-induced facilitation of the flexor reflex by M-35 in rats with intact and sectioned sciatic nerves

	% increase in reflex	
Nerve	CS control	CS after M-35
Intact	185.7 ± 47.1	296.7 ± 50.9*
Sectioned	179.3 ± 46.3	518.8 ± 80.7*†

CS was delivered 5–10 min after the i.t. M-35 to allow the facilitatory effect of M-35 to subside. The effect of the CS before and after 300 pmol of i.t. M-35 was expressed as percentage increase in reflex magnitude compared to baseline. Data are presented as mean ± SEM ($n = 6$). *, $P < 0.01$, compared to control response with Wilcoxon signed-rank test; †, $P < 0.05$, compared to the effect of CS after M-35 in rats with intact sciatic nerves with Mann–Whitney *U* test.

The endogenous inhibitory function of galanin in the somatosensory system demonstrated in the present study supports an inhibitory action of galanin in the central and peripheral nervous systems (for review, see ref. 31). An inhibitory action of galanin was further supported by studies at the molecular level in which it was found that the galanin receptor is coupled to an inhibitory guanine nucleotide binding protein (32–34) and activation of the galanin receptor may lead to closure of a voltage-dependent Ca^{2+} channel, opening of an ATP-sensitive K^+ channel, inhibition of adenylate cyclase activity, and inhibition of inositol trisphosphate production, events associated with inhibition of neuronal function (32–38).

In conclusion, our previous (6–8) and present studies suggest that neuropeptide galanin plays an inhibitory role in spinal nociception and this role may be enhanced after peripheral nerve injury. Consequently, galanin and galanin receptor agonists may be potential analgesic drugs.

We thank the Swedish Medical Research Council (07913, 04X-2887), the Bank of Sweden Tercentenary Foundation, the Wenner-Gren Center Foundation, Astra Pain Control AB, National Institute of Mental Health, Konung Gustav V:s och Drottning Victorias Stiftelse, Macus och Amalia Wallenbergs Minnesfond, and Marianne och Marcus Wallenbergs Stiftelse for financial support.

1. Tatemoto, K., Rökaeus, Å., Jörnvall, H., McDonald, T. L. & Mutt, V. (1983) *FEBS Lett.* **164**, 124–128.

2. Ch'ng, J. L. C., Christofides, N. D., Anand, P., Gibson, S. J., Allen, Y. S., Su, H. C., Tatemoto, K., Morrison, J. F. B., Polak, J. M. & Bloom, S. R. (1985) *Neuroscience* **16,** 343–354.
3. Skofitsch, G. & Jacobowitz, D. M. (1986) *Brain Res. Bull.* **15,** 191–195.
4. Melander, T., Hökfelt, T. & Rökaeus, Å. J. (1986) *Comp. Neurol.* **248,** 475–517.
5. Kuraishi, Y., Kawamura, M., Yamaguchi, H., Houtani, T., Kawabata, S., Futaki, S., Fujji, N. & Satoh, M. (1991) *Pain* **44,** 321–324.
6. Wiesenfeld-Hallin, Z., Villar, M. J. & Hökfelt, T. (1989) *Brain Res.* **486,** 205–213.
7. Xu, X.-J., Wiesenfeld-Hallin, Z., Villar, M. J. & Hökfelt, T. (1990) *Eur. J. Neurosci.* **2,** 733–743.
8. Xu, X.-J., Wiesenfeld-Hallin, Z. & Hökfelt, T. (1991) *Brain Res.* **541,** 350–353.
9. Yanagisawa, M., Yagi, N., Otsuka, M., Yanaihara, C. & Yanaihara, N. (1986) *Neurosci. Lett.* **70,** 278–282.
10. Nussbaumer, J. C., Yanagisawa, M. & Otsuka, M. (1989) *Br. J. Pharmacol.* **98,** 773–782.
11. Post, C., Alari, L. & Hökfelt, T. (1988) *Acta Physiol. Scand.* **132,** 583–584.
12. Randic, M., Gerber, G., Ryu, P. D. & Kangrga, I. (1986) *Soc. Neurosci. Abstr.* **13,** 1308.
13. Giuliani, S., Amann, R., Pagini, A. M., Maggi, C. A. & Meli, A. (1989) *Eur. J. Pharmacol.* **163,** 91–96.
14. Xu, X.-J., Hao, J.-X., Wiesenfeld-Hallin, Z., Håkanson, R., Folkers, K. & Hökfelt, T. (1991) *Neuroscience* **3,** 731–737.
15. Jessell, T. M., Tsunoo, A., Kanazawa, I. & Otsuka, M. (1979) *Brain Res.* **168,** 247–259.
16. McGregor, G. P., Gibson, S. J., Sabate, I. M., Blank, M. A., Christofides, N. D., Wall, P. D., Polak, J. M. & Bloom, S. R. (1984) *Neuroscience* **13,** 207–216.
17. Shehab, S. A. S. & Atkinson, M. E. (1986) *Brain Res.* **372,** 37–44.
18. Hökfelt, T., Wiesenfeld-Hallin, Z., Villar, M. J. & Melander, T. (1987) *Neurosci. Lett.* **83,** 217–220.
19. Villar, M. J., Cortés, R., Theodorsson, E., Wiesenfeld-Hallin, Z., Schalling, M., Fahrenkrug, J., Emson, P. & Hökfelt, T. (1989) *Neuroscience* **33,** 587–604.
20. Villar, M. J., Wiesenfeld-Hallin, Z., Xu, X.-J., Theodorsson, E., Emson, P. & Hökfelt, T. (1991) *Exp. Neurol.* **112,** 29–39.
21. Hökfelt, T., Verge, V. M. K., Wiesenfeld-Hallin, Z. & Eriksson, M. (1991) *Soc. Neurosci. Abstr.* **17,** 439.
22. Wiesenfeld-Hallin, Z., Xu, X.-J., Håkanson, R., Feng, D.-M. & Folkers, K. (1990) *Neurosci. Lett.* **116,** 293–298.
23. Wiesenfeld-Hallin, Z., Xu, X.-J., Villar, M. J. & Hökfelt, T. (1989) *Neurosci. Lett.* **105,** 149–154.
24. Langel, U., Land, T. & Bartfai, T. (1992) *Int. J. Pept. Protein Res.*, in press.
25. Bartfai, T., Bedecs, K., Land, T., Langel, Ü., Bertorelli, R., Girotti, P., Consolo, S., Xu, X.-J., Wiesenfeld-Hallin, Z., Nilsson, S., Pieribone, V. A. & Hökfelt, T. (1991) *Proc. Natl. Acad. Sci. USA*, **88,** 10961–10965.
26. Steranka, L. R., Manning, D. C., DeHaas, C. J., Ferkany, J. W., Borosky, S. A., Connor, J. R., Vaverk, R. J., Stewart, J. M. & Snyder, S. H. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3245–3249.
27. Wall, P. D. & Woolf, C. J. (1984) *J. Physiol.* **356,** 443–458.
28. Land, T., Langel, Ü., Fisone, G., Bedecs, K. & Bartfai, T. (1991) in *Methods in Neurosciences*, ed. Conn, P.M. (Academic, San Diego), Vol. 5, pp. 224–234.
29. Lindskog, S., Ahrén, B., Land, T., Langel, Ü. & Bartfai, T. (1992) *Eur. J. Pharmacol.* **210,** 183–188.
30. Wall, P. D. & Gutnick, M. (1974) *Exp. Neurol.* **43,** 580–593.
31. Hökfelt, T., Bartfai, T., Ceccatelli, S., Cortés, R., Fisone, G., Hulting, A.-L., Langel, Ü., Meister, B., Melander, T., Pieribone, V., Schalling, M., Verge, V. M. K., Villar, M., Wiesenfeld-Hallin, Z., Xu, X.-J. & Zhang, X. (1992) *Biomed. Res.* Suppl., in press.
32. Amiranoff, B., Lorinet, A. M. & Laburthe, M. J. (1989) *Biol. Chem.* **264,** 20714–20717.
33. Fisone, G., Langel, Ü., Carlquist, M., Bergman, T., Consolo, S., Hökfelt, T., Undén, A., Andell, S. & Bartfai, T. (1989) *Eur. J. Biochem.* **181,** 269–276.
34. Bedecs, K., Langel, Ü., Bartfai, T. & Wiesenfeld-Hallin, Z. (1992) *Acta Physiol. Scand.*, in press.
35. Ahrén, B., Arkhammar, P., Berggen, P.-O. & Nilsson, T. (1986) *Biochem. Biophys. Res. Commun.* **140,** 1059–1063.
36. Dunne, M. J., Bullett, M. J., Li, G. D., Wollheim, C. B. & Petersen, O. H. (1989) *EMBO J.* **8,** 413–420.
37. Konopka, L. M., McKeon, T. W. & Parsona, R. L. (1989) *J. Physiol.* **410,** 107–122.
38. Sharp, G. W., Le, M., Nrustel, Y., Yada, T., Russo, L. L., Bliss, C. R., Cormont, M., Monge, L. & Van, O. E. (1989) *J. Biol. Chem.* **264,** 7302–7309.