Activity of Centrally Administered Galanin Fragments on Stimulation of Feeding Behavior and on Galanin Receptor Binding in the Rat Hypothalamus

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Synthetic fragments of galanin 1-29 were administered intraventricularly or into the paraventricular nucleus of the hypothalamus for analysis of the critical amino acid sequence necessary to stimulate feeding behavior in rats. Galanin 1-29 and galanin fragment 1-16 significantly increased feeding at doses of 6 nmol microinjected into the lateral ventricles and 1 nmol microinjected into the hypothalamus. There was no significant effect of D-TRP² galanin 1-16 microinjected into the hypothalamus, and no significant effect of galanin fragments 1-9, 10-20, 12-29, 17-29, or 21-29 microinjected intraventricularly, on food consumption. Synthetic fragments of galanin 1-29 were assayed for displacement of ¹²⁵I-galanin 1-29 binding to rat hypothalamic membranes. The efficacies of the galanin fragments in the feeding paradigm were consistent with the relative affinities of these fragments for the hypothalamic galanin receptor in equilibrium binding experiments. The first 16 N-terminal amino acids appear to contain galanin agonist activity on increasing food consumption and to bind to the galanin receptor in the rat hypothalamus.

Galanin is a 29-amino acid peptide isolated from the porcine intestine (Tatemoto et al., 1983) and localized in the mammalian CNS (Skofitsch and Jacobowitz, 1985; Melander et al., 1986). Physiological actions of peripherally administered galanin include contraction of smooth muscle (Ekblad et al., 1989), activation of an ATP-dependent potassium channel (de Weille et al., 1988), and inhibition of glucose-stimulated insulin release (McDonald et al., 1985; Leonhardt et al., 1989). Galanin has been found to elevate circulating levels of growth hormone (Ottlecz et al., 1988; Murakami et al., 1987) and prolactin (Koshiyama et al., 1987), to inhibit dopamine release from the median eminence (Nordström et al., 1987), to inhibit ACh release from the hippocampus (Fisone et al., 1987), and to inhibit norepinephrine release from the hypothalamus (Tsuda et al., 1989). Other reported central effects of galanin include inhibition of slow cholinergic excitatory postsynaptic potentials in the hippocampus (Dutar et al., 1989), inhibition of locus coeruleus

firing (Seutin et al., 1989), inhibition of noradrenergic stimulation of cAMP production in the cerebral cortex (Nishibori et al., 1988), inhibition of carbachol-stimulated phosphatidyl inositol hydrolysis in the ventral hippocampus (Palazzi et al., 1988), and modulation of binding to the serotonin 1A receptor in the ventral limbic cortex (Fuxe et al., 1988). These diverse findings implicate galanin in inhibitory modulation of several central neurotransmitters, in initiating the release of pituitary hormones, and in the regulation of food consumption and metabolism both centrally and peripherally.

Behavioral actions of centrally administered galanin include inhibition of ACh-induced improvement of performance on a t-maze delayed alternation task in rats with basal forebrain lesions (Mastropaolo et al., 1988), inhibition of acquisition of a Morris swim-maze task (Sundström et al., 1988), inhibition of head twitches induced by a serotonin-2 agonist (Ögren and Fuxe, 1989), induction of feeding (Kyrkouli et al., 1986), and inhibition of the flexor spinal reflex (Wiesenfeld-Hallin et al., 1989).

Galanin thus appears to have a variety of biological actions at several anatomical sites in the brain and in the periphery (Rökäus, 1987). It would be of interest, therefore, to determine the amino acid sequence within galanin 1-29 that is critical for its biological activity and to determine whether this sequence differs at central versus peripheral sites. Induction of feeding provides a useful screen for evaluating the central activity of putative galanin analogues and antagonists. Porcine galanin 1-29 microinjected into the paraventricular nucleus of the hypothalamus (PVN) has been demonstrated to stimulate food consumption in satiated rats (Kyrkouli et al., 1986). Galanin appears to increase preferentially the consumption of a high-fat diet, as compared to a high-protein or a high-carbohydrate diet (Tempel et al., 1988). These actions of galanin in the PVN may be mediated through stimulation of norepinephrine release, because galanin microinjected into the PVN significantly increased extracellular norepinephrine in the PVN (Kyrkouli et al., 1988, 1990). The present study was designed to analyze a series of fragments of galanin 1-29 on the induction of feeding after central administration to the laboratory rat, and on displacement of ¹²⁵I-galanin 1-29 binding in rat hypothalamic membranes, in a first attempt to identify the amino acid sequence within galanin 1-29 that delineates the behavioral activity of this neuropeptide.

Materials and Methods

Animals. Male Sprague-Dawley rats, 250–300 gm, were housed in a temperature- and humidity-controlled vivarium, lights on 7 AM through

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7 PM, with rat chow and water available ad libitum. On the evening before the first feeding experiment, each rat was given 1 Nilla wafer cookie (Nabisco Brands, Inc., East Hanover, NJ) soaked in 5 ml distilled water, in a plastic weighing cup, which remained in the home cage overnight. Cookie mash was chosen as a highly palatable food, high in fat content, which rats consume readily after a single overnight habituation, to prevent food neophobia (Crawley and Kiss, 1985).

Surgery. Implantation of indwelling guide cannulae was performed under chloral hydrate anesthesia, using stereotaxic coordinates according to Paxinos and Watson (1986). Intraventricular coordinates were 1.0 mm caudal and 1.0 mm lateral to the bregma and 3 mm ventral to the surface of the skull, cannulated unilaterally. Coordinates for the paraventricular nucleus of the hypothalamus were 1.8 mm caudal and 0.4 mm lateral to the bregma, 7.0 mm ventral to the skull surface, mouthbar at -3.2 mm, cannulated bilaterally. One week after surgery, microinjections were performed through a 31-gauge stainless-steel hypodermic injection tube extending 1.0 mm below the ventral surface of the guide cannula. Unilateral intraventricular injections were 5 μ l vol, 1 μ l every 5 sec, with the injection tube remaining in place for an additional 40 sec. Bilateral PVN injections were 0.3 μ l vol, over a 47-sec period, using a Sage microinfusion pump (Orion Instruments, Cambridge, MA), with the injection tube remaining in place for an additional 30 sec.

Feeding test. Immediately after injection, the rat was placed in a clean cage containing 2 Nilla wafer cookies soaked in 10 ml distilled water, in a plastic weighing cup, for a 30-min period. The weight of the cookie mash was recorded before and after the 30-min feeding session. Consumption was calculated as the difference in weights before and after the session.

Statistical analysis. Food consumption was evaluated for each dose of each galanin fragment in comparison to that obtained after microinjection of vehicle. Statistical significance of peptide effects within a given series of vehicle, galanin, and fragments was evaluated by 1-way analysis of variance (ANOVA), followed by Newman-Keuls or Dunnett's test for significance of individual means. Each animal was used twice, with 1 week between the first and second uses and with treatments randomized such that each animal received a different treatment on the first and on the second use.

Histological analysis. At the end of each experiment, rats cannulated in the PVN were killed for histological analysis of the location of the injection sites. Brains were removed, stored in 8% formalin, sectioned at 100 μ m, stained with thionin, and analyzed by light microscopy. Data from animals with one or both cannulae outside of the PVN were eliminated from statistical analyses. Some brains from animals cannulated in the lateral ventricle were removed and similarly analyzed histologically for location of the injection site.

Peptides. Galanin 1-29, porcine and rat, was obtained from Bachem Bioscience Inc. (Philadelphia, PA) and from Bachem Fine Chemicals (Torrance, CA). Porcine and rat galanin 1-29 from both suppliers were tested intraventricularly and found to be equivalent in stimulation of feeding.

Fragments of galanin 1-29 were prepared by enzymatic cleavage or by solid-phase synthesis. Galanin fragments 1-9 and 10-20 were prepared by tryptic digestion of synthetic porcine galanin 1-29 (Bachem, Torrance, CA). Fragments 1-9 and 10-20 were obtained after digesting 700 μ g galanin 1-29 in 1 ml of 50 mM Tris-HCl (pH, 8.0) with 14 μ g DPCC trypsin (Sigma Chemical Co., St. Louis, MO) for 4 hr at 37°C. Galanin 1-16 was prepared by endoproteinase Asp-N digestion as previously described (Fisone et al., 1989a). Peptides were purified by reverse-phase (C-18) high-pressure liquid chromatography and characterized by amino acid sequence analysis.

Galanin fragments 12-29, 17-29, 21-29, a second batch of galanin 1-16, and D-TRP² 1-16 were synthesized manually on *p*-methylbenzhydrylamine resin (0.35 mmol/gm). Protected *t*-butoxycarbonyl amino acids were serine (benzyl-), threonine (benzyl-), tyrosine (*p*-bromobenzyloxycarbonyl-), histidine (dinitrophenyl-), and D- and L-tryptophan (formyl-). Asparagine and glutamine were coupled as active hydroxybenzotriazole esters. All other amino acids were activated by *N,N'*-dicyclohexylcarbodiimide in a molar ratio of 1:1. The protocol for stepwise solid-phase peptide synthesis using the *t*-butoxycarbonyl-benzyl protective group strategy has been published elsewhere (Tam and Merrifield, 1985). Removal of protection was carried out by low trifluoromethanesulfonic acid (Tam, 1985), and the resin was washed with trifluoroacetic acid (2 $\times$ 1 min), dichloromethane (3 $\times$ 1 min), dimethylformamide (3 $\times$ 1 min), ethanol (2 $\times$ 1 min), and diethyl ether (1 $\times$

1 min). The resin was dried under vacuum followed by final removal of protection by hydrogen fluoride containing 8% *p*-cresol and 2% ethanedithiol at 0°C for 40 min. Purification of the cleaved peptides was carried out by high-pressure liquid chromatography on a C-18 reverse-phase column.

For intraventricular experiments, galanin 1-29 and fragments were dissolved in a vehicle of 0.9% physiological saline. For PVN experiments, galanin 1-29 and fragments were dissolved in Ringer's vehicle (S. E. Kyrkouli and S. F. Leibowitz, personal communication). For all experiments with galanin and fragments, lyophilized peptide was dissolved, aliquoted, and stored at -20°C. Each aliquot was defrosted only on the day of use, and the excess was discarded.

Preparation of the radioligand ¹²⁵I-galanin. Preparation of the radioligand ¹²⁵I-galanin was performed by iodination of porcine galanin at room temperature by the chloramine-T method. Galanin, 10 μ g, in 20 μ l 0.05 M sodium phosphate buffer (pH 7.5) and 20 μ l chloramine-T (5 mg/ml) were added in a batch containing 2 mCi Na¹²⁵I (245 mCi/ml). The reaction was terminated by adding 100 μ l of a solution of sodium metabisulfite (1.2 mg/ml). The reaction mixture was transferred onto a column packed with SP-Sephadex C25, equilibrated with a solution of 100 mg/ml BSA, then washed and equilibrated with 0.05 M sodium phosphate buffer (pH, 5.0). The excess Na¹²⁵I was first eluted with a 0.05 M sodium phosphate buffer (pH, 5.0), while the iodinated galanin was eluted as a single peak with 0.05 M sodium phosphate buffer (pH, 8.1). Fractions corresponding to a peak of ¹²⁵I-galanin were pooled, the pH adjusted to 6.0 with acetic acid, and the aliquots stored at -18°C until use.

Preparation of membranes. Rat brains were quickly removed and the hypothalamus dissected for preparation of membranes. The tissue was homogenized (10% mass/vol) in 0.32 M sucrose buffered with 5 mM HEPES (pH, 7.4). The homogenate was diluted 10-fold and centrifuged at 1000 $\times$ g for 10 min. The supernatant was centrifuged at 10,000 $\times$ g for 45 min, and the pellet was resuspended in 5 mM HEPES-buffered Krebs-Ringer solution (pH, 7.4). All displacement curves were comprised of 24 or more independent observations taken at 8 different concentrations. Protein concentration was determined according to the Lowry method, using BSA as a standard.

Ligand-binding studies. Displacement experiments were carried out in a final volume of 400 μ l 5 mM HEPES/Krebs-Ringer solution, 0.05% BSA (pH, 7.4), containing 1 mM porcine ¹²⁵I-galanin and 70-100 μ g membrane preparation. Samples were incubated for 30 min at 37°C. Incubation was terminated by the addition of 10 ml ice-cold HEPES/Krebs-Ringer solution, followed by rapid filtration over Whatman GF/B filters, precoated for 5-6 hr in 0.3% polyethyleneimine solution. Specific binding was defined as that displaceable by 1 μ M rat galanin 1-29.

Results

Figure 1 shows the effects of intraventricularly administered galanin 1-29 and N-terminal galanin fragments 1-9 and 1-16, at a dose of 6 nmol/5 μ l, on consumption of a palatable food. As compared to values obtained after saline vehicle alone, galanin 1-29 and galanin 1-16 significantly increased food consumption (ANOVA $F_{3,75} = 18.47$, $p < 0.001$; Newman-Keuls $p < 0.01$ for 1-29, $p < 0.05$ for 1-16, NS for 1-9).

Figure 2 shows the effects of intraventricularly administered galanin 1-29 and C-terminal galanin fragments 12-29, 17-29, 21-29, and 10-20, at a dose of 6 nmol/5 μ l, on food consumption. As compared to values obtained after saline vehicle alone, only galanin 1-29 significantly increased feeding (ANOVA $F_{5,69} = 7.74$, $p < 0.001$; Newman-Keuls $p < 0.01$ for 1-29, NS for 12-29, 17-29, 21-29, and 10-20).

Figure 3 illustrates the placement of bilateral cannulae and injection sites in the PVN. Data from 7 animals were eliminated from statistical analyses because of histological evidence for one or both injection sites being outside of the PVN.

Figure 4 shows the effects of galanin 1-29, galanin 1-16, and D-TRP²-galanin 1-16 on food consumption after microinjection into the PVN. As compared to values obtained after Ringer's vehicle alone, galanin 1-29 and galanin 1-16 significantly in-

Figure 1. Comparison of food consumption after intraventricular microinjection of saline, galanin 1-29, and N-terminal galanin fragments 1-9 and 1-16. Each peptide was administered at a dose of 6 nmol in a vol of 5 μ l over a 1-min period. Consumption of a palatable cookie mash over a 30-min period immediately after microinjection was significantly elevated after galanin 1-29 (**, $p < 0.01$) and after galanin 1-16 (*, $p < 0.05$), as compared to saline-vehicle control values. Data are expressed as mean $\pm$ SEM. Number of animals is shown in parentheses for each treatment group.

creased feeding (ANOVA $F_{6,29} = 18.17$, $p < 0.001$; Dunnett's test $p < 0.05$ for galanin 1-29 at a dose of 0.5 nmol, $p < 0.01$ for galanin 1-29 at 1 nmol, $p < 0.01$ for galanin 1-16 at 1 nmol). There was no significant effect of galanin 1-16 at the dose of 0.5 nmol, nor of D-TRP² 1-16 at 1 nmol. There was no significant difference in food consumption between animals receiving vehicle microinjection alone and animals receiving no microinjection.

Behavioral data from 7 animals in the PVN experiments were excluded from the statistical analysis presented above after histological analyses revealed cannulae placements outside of the PVN. Animals with both cannulae placements dorsal, ventral, or rostral to the PVN showed no increase in food consumption after microinjection of galanin 1-29. Animals with one cannula placed within the PVN but the other cannula medial or dorsal to the PVN showed partial responses to galanin 1-29 on food consumption.

Figure 5 shows the displacement of ¹²⁵I-galanin 1-29 from hypothalamic binding sites by increasing concentrations of galanin fragments. Galanin 1-16 displaced radioligand binding with a K_D of 8.2 nM. D-TRP² galanin 1-16, galanin 17-29, and galanin 21-29 did not displace radioligand binding in the nanomolar range of concentrations.

Discussion

The present data demonstrate that an N-terminal fragment of galanin, 1-16, stimulates food consumption in a manner similar to stimulation of food consumption by the full-length sequence, 1-29. When administered into the lateral ventricle, galanin fragment 1-16 was approximately 1/3 as potent as galanin 1-29. When administered into the PVN, galanin fragment 1-16 was approx-

Figure 2. Comparison of food consumption after intraventricular microinjection of saline, galanin 1-29, and C-terminal galanin fragments 12-29, 17-29, 21-29, and 10-20. Each peptide was administered at a dose of 6 nmol/5 μ l. Food consumption was assayed and analyzed as described in Figure 1. Consumption was significantly elevated only after galanin 1-29 (**, $p < 0.01$) as compared to saline-vehicle control values. Data are expressed as mean $\pm$ SEM. Number of animals is shown in parentheses for each treatment group.

imately half as potent as galanin 1-29. Binding experiments with hypothalamic membranes found that the rank order of efficacy of several galanin fragments for displacement of ¹²⁵I-galanin 1-29 binding coincided with the rank order of their efficacy in inducing food consumption after PVN microinjection. These results support an interpretation that the actions of galanin 1-29 and galanin 1-16 on feeding behavior are mediated by the hypothalamic galanin receptor.

Substitution of a D-TRP for the L-TRP amino acid in the number 2 position of galanin 1-16 produced a relatively inactive fragment in both the feeding behavior and the receptor binding assays, suggesting that the second amino acid at the N-terminal is critical for biological activity in the present paradigms. Lack of activity of the 1-9 fragment on feeding behavior suggests that the minimum amino acid sequence necessary for biological activity in the present paradigm is longer than the first 9 amino acids. Lack of activity of the 10-20, 12-29, 17-29, and 21-29 fragments on feeding behavior, at doses equimolar to the active dose of 1-29 and of 1-16, suggests that the C-terminal end of galanin 1-29 is less critical for induction of feeding behaviors.

Another explanation for the lack of activity of some of the galanin fragments might be a rapid metabolic degradation *in vivo*. In the case of the active fragment, 1-16, where the increase in feeding was less than the increase in feeding after galanin 1-29 at comparable molar doses, reduced potency could reflect a more rapid breakdown of 1-16 after microinjection. The increase in feeding after 1-16 was greater after administration into the PVN than after administration into the lateral ventricles, possibly indicating degradation in the cerebrospinal fluid as the peptide traveled from the lateral ventricle to the hypothalamus. Alternatively, the lesser effects of 1-16 could reflect a reduced efficacy. C-terminal portions of the tertiary structure of galanin 1-29 may be necessary for initiation of the maximal biological

Figure 3. Representative photomicrograph of bilateral cannula placements in PVN. Arrows indicate microinjection sites. *F*, fornix; *3V*, third ventricle. Magnification, $31\times$.

response at the central galanin receptor. A third possibility that may explain the apparently lower efficacy of fragment 1-16 is that the N-terminal fragment is more hydrophobic than the full-length peptide, and may be adsorbed nonspecifically. This greater hydrophobicity may make the N-terminal fragments interesting with respect to penetration into the brain when tested by systemic administration.

The finding that galanin 1-16 is an agonist in the hypothalamus is consistent with the recent report by Fisone et al. (1989a) that galanin 1-16 displaced ^{125}I -labeled galanin binding in the rat ventral hippocampus and spinal cord, that galanin 1-16 inhibited carbachol-stimulated phosphatidyl inositol hydrolysis

in the ventral hippocampus, and that intraventricularly administered galanin 1-16 inhibited scopolamine-induced release of ACh from the ventral hippocampus *in vivo*. In addition, substitution of L-TRP² by D-TRP² in galanin 1-16 produced a loss in activity in displacement of ^{125}I -galanin binding in the ventral hippocampus (Fisone et al., 1989a), consistent with the present behavioral and receptor-binding data in the hypothalamus.

Bioassays in the PNS have shown a profile of galanin-fragment activity with similarities to the present data. N-terminal activity has been shown by galanin fragments 1-10 (Ekblad et al., 1989) and 1-20 (Fox et al., 1988) on smooth muscle contraction, by 2-29 and 1-15 on insulin release from the pancreas (Amiranoff et al., 1989; Hermansen et al., 1989), and by 2-29 and 1-15 on displacement of binding to pancreatic galanin receptors (Hermansen et al., 1989; Lagny-Pourmir et al., 1989). In these studies, fragments lacking the first 2 amino acids from the N-terminal (e.g., 3-29) were without effect (Amiranoff et al., 1989; Hermansen et al., 1989), and fragments from the C-terminal of the peptide, including 9-29, 10-29, and 15-29, were relatively inactive (Amiranoff et al., 1989; Hermansen et al., 1989; Rossowski and Coy, 1989). Studies with galanin fragments in the CNS have shown some activity in displacing ^{125}I -galanin 1-29 binding at micromolar concentrations by C-terminal fragments such as 12-29, 15-29, 18-29, and 21-29, though N-terminal fragments such as 1-15 and 1-16 were considerably more potent (Gabriel et al., 1988; Fisone et al., 1989b; Lagny-Pourmir et al., 1989). Potential differences in galanin fragments that are active in the peripheral versus the central nervous systems may indicate differences in galanin-receptor subtypes in the periphery versus in the brain and/or different routes of metabolism of galanin and its fragments in the periphery versus the brain. It is also interesting to speculate that the peripheral action of gal-

Figure 4. Comparison of food consumption after microinjection of Ringer's solution, galanin 1-29, galanin 1-16, or galanin D-TRP² 1-16 into the PVN. Consumption of cookie mash over a 30-min period was significantly elevated after galanin 1-29 (*, $p < 0.05$ at a dose of 0.5 nmol; **, $p < 0.01$ at 1.0 nmol) and after galanin 1-16 (*, $p < 0.05$ at 1.0 nmol), as compared to Ringer's vehicle control values. A lower dose of galanin 1-16 (0.5 nmol) and the D-TRP²-substituted galanin 1-16 at 1.0 nmol had no significant effect on food consumption. Data are expressed as mean $\pm$ SEM. Number of animals is shown in parentheses for each treatment group.

Figure 5. Displacement of ^{125}I -galanin (porcine, 1 nM) from binding sites in membranes from rat hypothalamus by galanin 1-29 (solid circles) and by galanin fragments 1-16 (open circles), D-TRP 2 1-16 (open squares), 17-29 (open triangles), and 21-29 (solid triangles). Each point is measured in triplicate.

anin in inhibiting glucose-stimulated insulin release (McDonald et al., 1985; Leonhardt et al., 1989) may be functionally linked to the central action of galanin in stimulating consumption of a high-fat diet (Tempel et al., 1988).

In conclusion, data from a behavioral assay of galanin fragments, from the induction of feeding after central administration, and from binding data using rat hypothalamic membranes suggest that the N-terminal 16 amino acids contain agonist activity at the hypothalamic galanin receptor. This finding may provide useful information for the development of potent, selective galanin agonists and antagonists to further investigate the role of endogenous galanin in biological functions.

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