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Galanin inhibits acetylcholine release in the ventral hippocampus of the rat: Histochemical, autoradiographic, *in vivo*, and *in vitro* studies

(coexistence/neuropeptide/autoreceptor/evoked release)

GILBERTO FISONE*†, CHUN F. WU*, SILVANA CONSOLO*, ÖIE NORDSTRÖM†, NICLAS BRYNNE†, TAMAS BARTFAI†, TOR MELANDER‡, AND TOMAS HÖKFELT‡§

*Istituto di Ricerche Farmacologiche "Mario Negri," 20157 Milano, Italy; †Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden; and ‡Department of Histology, Karolinska Institute, S-104 05 Stockholm, Sweden

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ABSTRACT **A high density of galanin binding sites was found by using ^{125}I-labeled galanin, iodinated by chloramine-T, followed by autoradiography in the ventral, but not in the dorsal, hippocampus of the rat. Lesions of the fimbria and of the septum caused disappearance of a major population of these binding sites, suggesting that a large proportion of them is localized on cholinergic nerve terminals of septal afferents. As a functional correlate to these putative galanin receptor sites, it was shown, both *in vivo* and *in vitro*, that galanin, in a concentration-dependent manner, inhibited the evoked release of acetylcholine in the ventral, but not in the dorsal, hippocampus. Intracerebroventricularly applied galanin (10 μg/15 μl) fully inhibited the scopolamine (0.5 mg/kg, s.c.)-stimulated release of acetylcholine in the ventral, but not in the dorsal, hippocampus, as measured by microdialysis technique. *In vitro*, galanin inhibited the 25 mM K^+-evoked release of [^{3}H]acetylcholine from slices of the ventral hippocampus, with an IC_{50} value of ≈50 nM. These results are discussed with respect to the colocalization of galanin- and choline acetyltransferase-like immunoreactivity in septal somata projecting to the hippocampus.**

Galanin, a 29-amino acid peptide with a C-terminal amide (1), has a wide and specific distribution in the central nervous system, as demonstrated by immunohistochemical (2–5) and radioimmunological (3, 6) techniques. Extensive overlap of galanin-like immunoreactivity and cholinergic markers such as acetylcholinesterase (AcChoE) staining and choline acetyltransferase-like immunoreactivity has been demonstrated in septal cell bodies and hippocampal fibers of the rat (7, 8) and owl monkey (9), suggesting an involvement of galanin in cholinergic neurotransmission (7–9). Galanin has a direct, contractile effect on some smooth muscle preparations (10, 11), and it acts as an inhibitory transmitter when applied to myenteric neurons (12). Galanin also has potent inhibitory effects on the release of dopamine from rat median eminence (13) and on the release of acetylcholine (AcCho) in the guinea pig taenia coli (10, 11).

The present paper describes the existence of galanin binding sites in the ventral hippocampus. It further demonstrates, *in vivo* and *in vitro*, that one of the effects of galanin, which may be exerted through these sites, involves inhibition of the evoked release of AcCho.

MATERIALS AND METHODS

Animals. Adult male Sprague–Dawley rats (180–250 g) were used in the autoradiographic, immunohistochemical, histochemical, and *in vitro* experiments. Adult female CD-COBS rats (180–250 g) were used in the *in vivo* experiments.

Chemicals and Antisera. Galanin (lot no. 18186) was purchased from Bachem (Bubendorf, Switzerland). [^{3}H]-Choline (2.96 TBq/mmol) and Na[^{125}I] were purchased from Amersham. Scopolamine hydrobromide was purchased from Aldrich; physostigmine sulfate was from Sigma. All the other chemicals used were of analytical grade. Galanin antiserum was purchased from Peninsula Laboratories (San Carlos, CA). Secondary antisera were purchased from Boehringer Mannheim (Stockholm).

Surgical Procedures. Four rats were anesthetized with chloral hydrate. Lesions of the dorsal hippocampal afferents were performed by the use of a retractable knife. The knife was lowered into the brain (coordinates in relation to bregma: AP, −1.4; ML, 3.0; DV, −4.0), and the blade was pushed out to the midline and retracted through the brain.

Immunohistochemical, Histochemical, and Autoradiographic Analyses. Two rats were perfused through the ascending aorta with a picric acid/formalin mixture (14) for 6 min. The brains were postfixed in the same fixative, rinsed, and sectioned on a cryostat. Sections were incubated overnight at 4°C with galanin antiserum (1:400 dilution), rinsed, incubated with fluorescein isothiocyanate-conjugated secondary antibody (1:40 dilution) at 37°C for 30 min (15), rinsed, mounted, and examined with a fluorescence microscope. Some sections were processed for demonstration of AcChoE, either by the method of Karnovsky and Roots (16) or by immunohistochemistry using rabbit polyclonal antibody to AcChoE (17). For the autoradiographic analysis of ^{125}I-labeled galanin binding sites, the procedure of Young and Kuhar (18) was used. Briefly, pig galanin was iodinated with Na[^{125}I] by the chloramine-T method and purified on an ion-exchange column. Rats were perfused with ice-cold Tyrode's solution, and the brain was sectioned on a cryostat, followed by incubation with ^{125}I-labeled galanin for 45 min at room temperature. Sections were rinsed, dried by a stream of cold air, exposed to formalin vapors, and covered by tritium-sensitive film (Ultrofilm, LKB, Stockholm). For control of nonspecific binding, unlabeled galanin (1 μM) was added to the incubation medium.

***In Vivo* Experiments.** In the *in vivo* release experiments, a thin dialysis fiber was implanted, essentially as described by Ungerstedt (19) and Benveniste *et al.* (20), in the hippocampi of anesthetized rats. A looped dialysis probe was implanted vertically into the ventral hippocampus of one side, while a straight dialysis probe was inserted through both dorsal hippocampi. The day after implantation, the dialysis tube was

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Abbreviations: AcCho, acetylcholine; AcChoE, acetylcholinesterase.

§To whom reprint requests should be addressed.

perfused at a constant rate of 2 μl/min with Ringer's solution (147 mM NaCl/3.4 mM $CaCl_2$/4 mM KCl, pH 6.1) containing 10 μM physostigmine. The perfusate was discarded during the first 30 min and then collected at 20-min intervals. Endogenous AcCho collected in the samples was assayed by a sensitive and specific radioenzymatic method, as described by Consolo *et al.* (21).

***In Vitro* Experiments.** Rats were decapitated, the brain was dissected, and the hippocampi were divided into dorsal and ventral regions and sliced in sagittal direction to a thickness of 0.4 mm with a McIlwain tissue chopper. The slices were loaded with [^{3}H]choline (20 μCi/ml; 1 Ci = 37 GBq), during an incubation of 45 min, at 37°C, in Krebs–Ringer buffer (118 mM NaCl/5 mM KCl/1.2 mM $CaCl_2$/1.2 mM $MgSO_4$/1.2 mM NaH_2PO_4/25 mM $NaHCO_3$/11 mM glucose, pH 7.4), bubbled with 95% O_2/5% CO_2, then washed to remove excess of [^{3}H]choline, and placed into baskets with perforated bottoms (22). The baskets were subsequently immersed for 5-min periods (five times) into tubes containing Krebs–Ringer buffer (with 5 mM K^+) and then Krebs–Ringer buffer with 25 mM K^+ with and without drugs as specified. In experiments where [^{3}H]choline and [^{3}H]AcCho were sepa-

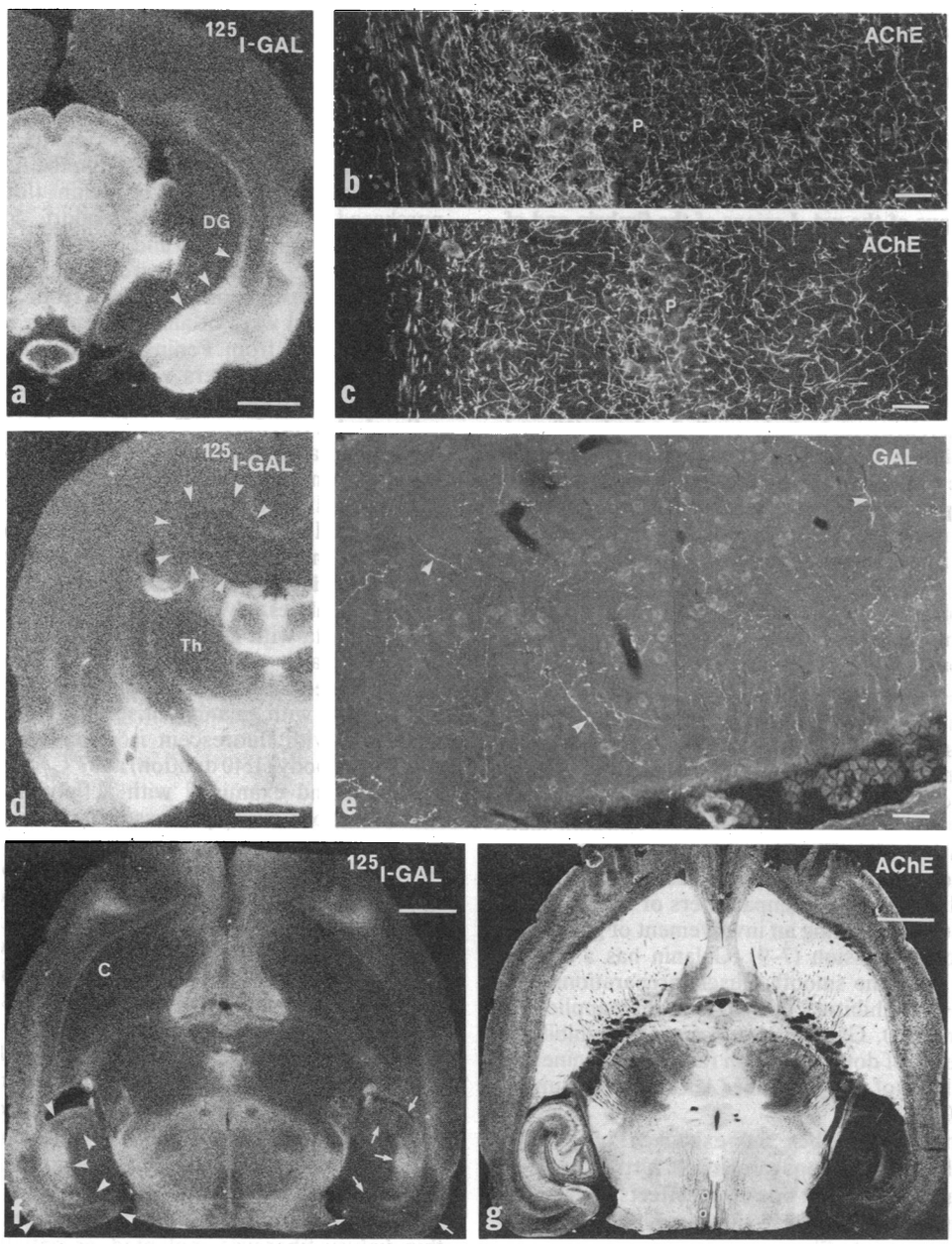

FIG. 1. Receptor autoradiograms showing ^{125}I-labeled galanin binding (^{125}I-GAL) (*a*, *d*, and *f*), immunofluorescence micrographs of sections of dorsal (*b*) and ventral (*c* and *e*) hippocampus after incubation with antiserum to AcChoE (AChE) (*b* and *c*) and to galanin (GAL) (*e*), and AcChoE staining (dark field) (*g*). The presence of ^{125}I-labeled galanin binding sites in the temporal (ventral) pole (arrowheads in *a*) of the hippocampal formation is seen (*a*) as compared to the apparent absence in the dorsal (septal) pole (arrowheads in *d*). A dense AcChoE-immunoreactive fiber plexus is seen both in the ventral (*b*) and dorsal (*c*) hippocampus, whereas galanin-positive fibers are preferentially seen in the ventral hippocampus but are present only in comparatively low numbers (*e*). A reduction in ^{125}I-labeled galanin binding (cf. arrows and arrowheads in *f*) is seen in the hippocampus, the subiculum, and the entorhinal cortex on the lesion side (to the right) after transection of the dorsal hippocampal afferents, but some binding sites remain. The extent of the lesion of the dorsal hippocampal afferents is shown with the help of AcChoE staining of an adjacent section (*g*). DG, dentate gyrus; P, pyramidal cell layer; Th, thalamus; C, caudate nucleus. (Bars = 1 mm in *a*, *d*, *f*, and *g* and 50 μm in *b*, *c*, and *e*).

rated, the buffer was complemented with 10 μM physostigmine. Separation of [^{3}H]choline and [^{3}H]AcCho was carried out by choline kinase–ion-pair extraction method (23, 24). Tissue content of [^{3}H]choline and [^{3}H]AcCho was determined following homogenization of the slices in 1 M NaOH overnight and measuring radioactivity in an aliquot in a scintillation spectrometer at 36% efficiency.

RESULTS

Specific binding of ^{125}I-labeled galanin was found in the ventral hippocampus (Fig. 1 *a* and *f*), while similar binding was virtually absent in the dorsal hippocampus (Fig. 1*d*). Lesions of the fimbria and of the septum caused a loss of specific ^{125}I-labeled galanin binding (Fig. 1*f*). The loss was most pronounced in the CA3 region, subiculum and entorhinal cortex, whereas a "patch" of binding sites in the CA1/CA2 region remained less affected by the lesion. The effect of the lesion on AcChoE staining is shown in Fig. 1*g* from a section adjacent to that in Fig. 1*f*. Although presumable cholinergic fibers, as visualized with antiserum to AcChoE, can be shown to be abundant both in the ventral (Fig. 1*b*) and dorsal (Fig. 1*c*) hippocampus, galanin-positive fibers can generally be visualized in lower numbers and in the ventral hippocampus (CA3 region) mainly (Fig. 1*e*).

The possibility that the putative galanin receptors are localized on cholinergic nerve terminals prompted experiments on the effect of galanin on the release of AcCho. *In vitro* studies showed that 25 mM K^+ depolarization-evoked release of [^{3}H]AcCho was inhibited by 1 μM galanin in slices from the ventral, but not from the dorsal, hippocampus (Table 1). The inhibitory effect of galanin on the K^+-evoked release of [^{3}H]AcCho from ventral hippocampal slices was concentration dependent (Fig. 2). Galanin, at ≈50 nM, produced half-maximal inhibition. Maximal inhibition (39%) of the evoked release was obtained at ≈500 nM galanin.

The inhibitory effect of galanin on AcCho release from the ventral, but not from the dorsal, hippocampus could also be demonstrated *in vivo* using the microdialysis technique (Table 2). The release of endogenous AcCho from the hippocampus was evoked by systemic administration of the muscarinic antagonist scopolamine (0.5 mg/kg, s.c.). This caused a time-dependent increase in the outflow of AcCho in dorsal and ventral hippocampus (Fig. 3). Galanin (10 μg/15 μl) fully inhibited the evoked release of AcCho from the ventral, but not from the dorsal, hippocampus (Fig. 3). Galanin (10 μg/15 μl), when applied intracerebroventricularly, caused a slight increase in the basal, nonscopolamine-induced release of AcCho (Fig. 3).

DISCUSSION

Immunohistochemical (2–5) and radioimmunological (3–6) methods have indicated the presence of high concentrations of galanin-like immunoreactivity in the hippocampus of rat and owl monkey, especially in the ventral part (7). The colocalization of galanin and cholinergic markers, such as

Table 1. Effect of 1 μM galanin on the 25 mM K^+- evoked release of [^{3}H]AcCho and [^{3}H]choline from slices of dorsal and ventral hippocampus of the rat

Tissue slice	Fractional release of [^{3}H]AcCho and [^{3}H]choline	
	No galanin	Galanin
Dorsal hippocampus	0.075 ± 0.003	0.073 ± 0.004
Ventral hippocampus	0.074 ± 0.004	0.059 ± 0.003*

The data are the means ± SEM of six animals. $P < 0.05$ (*), galanin vs. no galanin group by Student's *t* test.

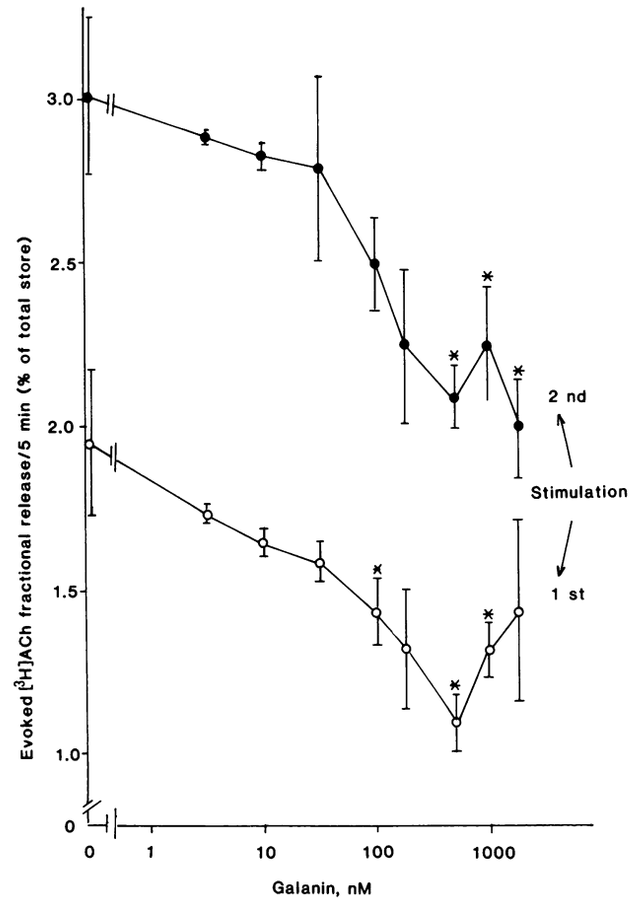

FIG. 2. Effect of various concentrations of pig galanin on the 25 mM K^+-evoked release of [^{3}H]AcCho ([^{3}H]ACh) from tissue slices of the rat ventral hippocampus. The data are expressed as the released fraction of the [^{3}H]AcCho stored in the tissue at the start of the experiments and are the means ± SEM of four to nine animals. $P < 0.05$ (*) for experimental value vs. respective control group by Student's *t* test.

choline acetyltransferase-like immunoreactivity and AcChoE staining in septal somata, suggested that some of the hippocampal galanin-like immunoreactivity may be colocalized with AcCho in septal afferents (7–9). On the basis of these morphological findings and on the basis of results indicating that galanin causes presynaptic inhibition of release of a classical transmitter in some systems (10, 11, 13), a possible presynaptic regulatory effect of galanin on AcCho seemed possible.

Whereas galanin-like immunoreactivity is present in ven-

Table 2. Effect of galanin on the scopolamine-induced release of AcCho from perfused dorsal and ventral hippocampus of the rat

Tissue	Scopolamine	AcCho release, fmol/min	
		No galanin	Galanin
Dorsal hippocampus	−	689 ± 43	691 ± 69
	+	2639 ± 260*	2498 ± 260
Ventral hippocampus	−	318 ± 53	463 ± 19
	+	938 ± 156†	434 ± 64

Galanin was injected (10 μg/15 μl, i.c.v.) 2 min before scopolamine. Data are the means ± SEM of the third 20-min fraction after galanin treatment (cf. Fig. 3). The values are corrected for recovery (49.2%). $P < 0.01$ (*) and $P < 0.05$ (†) for experimental group vs. respective no galanin/no scopolamine groups by Tukey's test for unconfounded means. In the ventral hippocampus the interaction between the two treatments is significant [$F(1,10) = 6.6$; $P < 0.03$ by the ANOVA two-way factorial analysis].

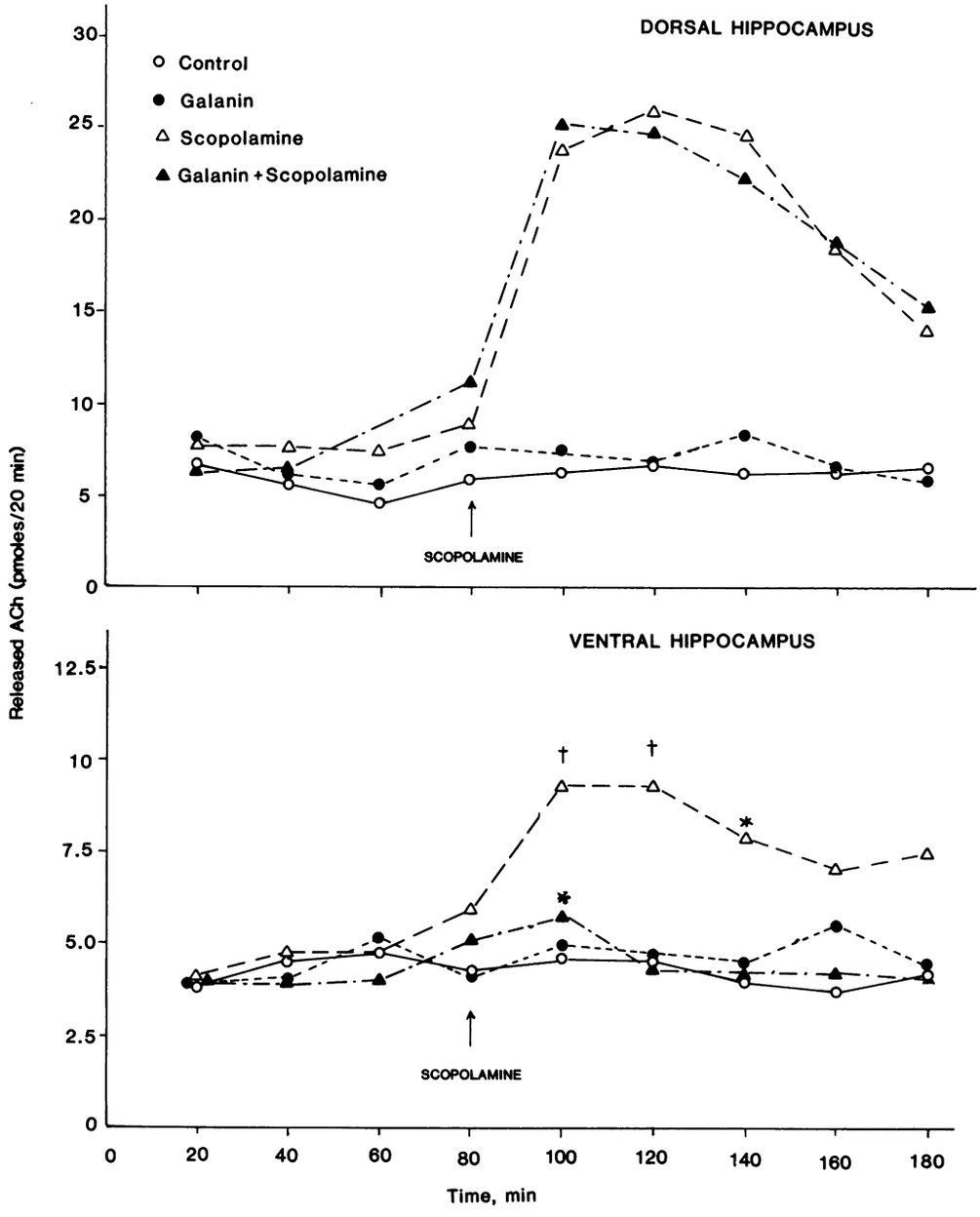

FIG. 3. Effect of galanin (10 μg/15 μl, i.c.v.) on the scopolamine (0.5 mg/kg, s.c.)-induced release of AcCho (ACh) as a function of time in dorsal and ventral hippocampus. Galanin was injected 2 min before scopolamine. Perfusate was collected for 1 hr (three fractions, 20 min for each fraction) before injection of galanin and/or scopolamine. The data are the means ± SEM of four animals. $P < 0.01$ (†) and $P < 0.05$ (*) for experimental vs. respective "no scopolamine" groups by Dunnett's test.

tral and dorsal hippocampus in comparable amounts as measured by RIA (4), the type of innervation differs (7–9), and the immunohistochemistry has shown a more prominent ventral innervation (5, 8). In fact, the nature of the galanin-positive fibers shown here in the ventral hippocampus is uncertain, since several types of afferents, as well as local neurons, seem to contain galanin-like immunoreactivity (4, 5). In contrast to the galanin fibers, AcChoE-immunoreactive fibers form a dense plexus in dorsal and ventral hippocampus. The present results show that high-affinity, specific binding sites for ^{125}I-labeled galanin could be found in the ventral, but not in the dorsal, hippocampus, in agreement with an autoradiographic binding study by Skofitsch *et al.* (25). The discrepancy between the presence of galanin-like immunoreactivity and the lack of ^{125}I-labeled galanin binding sites in the dorsal hippocampus may stem from limitations of the autoradiographic technique, which permits detection only of the high-affinity receptors. Thus, although autoradiography studies so far show that the dorsal hippocampus lacks high-affinity binding sites for ^{125}I-labeled galanin, this does not exclude the presence of other classes of galanin binding sites with other characteristics and/or binding sites in very low numbers.

The lesion experiments suggest that at least part of the ^{125}I-labeled galanin binding sites are localized on septal afferents to the hippocampus. The origin of some of these afferents is likely to be the septum and diagonal band nuclei, where also most of the cholinergic somata projecting to the hippocampus are located. The disappearance of the major part of ^{125}I-labeled galanin sites upon fimbria lesion and the colocalization of galanin-like immunoreactivity with cholinergic markers in the septal somata (7) strongly suggest that a population of ^{125}I-labeled galanin sites are on cholinergic septal afferents.

The function of the ^{125}I-labeled galanin binding sites or of the putative galanin receptors in the ventral hippocampus

was investigated with respect to the regulation of AcCho release from the septal afferents. In agreement with the autoradiographic data on distribution of galanin binding sites, galanin inhibited the evoked release of AcCho in the ventral, but not in the dorsal hippocampus. These data were obtained when studying release of [^{3}H]AcCho from [^{3}H]choline-labeled pools *in vitro* and when measuring release of endogenous AcCho *in vivo*. The inhibitory effects of galanin are concentration dependent and probably exerted at high-affinity receptors, since the half-maximal inhibition of AcCho release was observed at 50 nM galanin. The affinity is probably even higher, since the experiments were carried out using tissue slices with limited permeability for a 29-amino acid peptide. Also, galanin used here is purified from pig and differs in the amino acid sequence from that of the rat, a circumstance that may lead to lower-affinity binding of this ligand to galanin receptors in the rat brain.

The major regulation of AcCho release in the hippocampus involves a muscarinic feedback inhibition by AcCho (cf. refs. 26 and 27) exerted at autoreceptors on the terminals of septal afferents (28). As shown here, muscarinic antagonists produce substantial release *in vivo*, whereas muscarinic agonists inhibit both basal and evoked release by 30–40% (28). The galanin-produced inhibition of the evoked release of AcCho is as potent as the muscarinic feedback inhibition. *In vivo*, galanin fully blocked the scopolamine-enhanced release, which was most due to disinhibition of the muscarinic feedback by antagonist (26–28). A basic difference between the inhibitory actions of muscarinic agonists and galanin seems to be that the former are also potent in altering basal AcCho outflow, whereas galanin seems to inhibit only the evoked release and in our experiments did not affect the basal release. Thus, one may postulate that galanin [similarly to other peptide messengers that are likely to be released at higher frequencies of stimulation than classical neurotransmitters (cf. refs. 29 and 30)] is an effective feedback regulator of AcCho release in a different, higher-frequency range than AcCho itself. This situation may be an additional example of presynaptic interactions of coexisting neurotransmitters that regulate each other's release, as has been observed (cf. ref. 31) in several other systems with coexisting neurotransmitters.

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