Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 7742–7746, October 1990
Neurobiology

Effects of central nervous system lesions on the expression of galanin: A comparative *in situ* hybridization and immunohistochemical study

(acetylcholine/5-hydroxytryptamine/neuropeptide/plasticity/Alzheimer disease)

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Contributed by Tomas Hökfelt, July 2, 1990

ABSTRACT **We have used *in situ* hybridization and immunohistochemistry to study the expression of galanin mRNA and galanin-like immunoreactivity after decortication and lesions of the ventral hippocampus. After decortication the levels of both galanin mRNA and galanin-like immunoreactivity were increased in the dorsal raphe nucleus. In addition, in decorticated rats, but not in controls, galanin mRNA could be seen in dorsal and ventral nuclei of the thalamus and in the remaining parts of the cortex. Increases in galanin mRNA and galanin-like immunoreactivity were also observed in the septum-vertical diagonal band after electrocoagulation lesions of the ventral hippocampus. In contrast, no changes were found after ibotenic acid lesions of the same hippocampal area. These results suggest that increases in the expression of galanin occur in certain neuron populations after direct lesion of their axons and/or terminal fields.**

Galanin is a 29-amino acid peptide originally isolated from pig small intestine (1). Recently, cDNAs encoding the rat (2, 3) and the bovine (4) galanin have been cloned and sequenced. Immunohistochemical and radioimmunoassay studies have shown that galanin-like immunoreactivity (-LI) is widely distributed in the rat brain (5–7) and coexists with other neuropeptides and classical neurotransmitters within the central nervous system (8, 9), among others, with choline acetyltransferase (ChAT) in the septum-basal forebrain (8) and with 5-hydroxytryptamine (5-HT) in the dorsal raphe nucleus (9).

It has recently been shown that the synthesis of galanin can be upregulated after lesion. Thus, increases in galanin mRNA and galanin peptide have been observed in dorsal root ganglion neurons after transection of the sciatic nerve (10, 11) and in the magnocellular secretory neurons of the hypothalamic paraventricular and supraoptic nuclei after hypophysectomy (12). Also, intraventricularly administered colchicine markedly increases galanin mRNA in many central systems, including some cholinergic basal forebrain neurons (13). We have speculated that this upregulation may be related to a "lesion effect" (13). To directly test this hypothesis we have analyzed to what extent experimentally induced central nervous system lesions also may change galanin expression and compared mechanical lesions with excitotoxin lesions.

MATERIALS AND METHODS

Experimental Animals. Sixty male albino Sprague–Dawley rats (150–200 g) were used. Animals were anesthetized with chloral hydrate (350 mg/kg, i.p.) and placed in a stereotaxic instrument. Unilateral (right side) and bilateral decortications were performed, whereby the frontoparietal cortex was removed by aspiration. The cingulate cortex was left intact. After operation, the wounds were filled with Spongostan, and animals were allowed to survive for 4 days.

Ibotenic acid (IbA) (total 1.6 μl; 10 μg/μl) was injected into the ventral hippocampus at four sites [coordinates AP −2 and −4.6, ML +4.5 and +5.5 mm from bregma (14)] with a 1-μl Hamilton syringe. At each site the needle was lowered −7.5 mm from the brain surface and four injections of 0.1 μl of IbA solution each were given at 1-mm intervals from DV −7.5 to −4.5 mm. The animals were allowed to survive for 12 days. Cresyl violet staining of the tissue showed the absence of cell bodies in an area including the ventral hippocampus and entorhinal cortex.

Electrocoagulation lesions in the ventral hippocampus were made at the same four insertion points described for IbA injections. Two milliampere current stimulation was given at 1-mm intervals from DV −7.5 to −3.5 mm (10 sec each) from the brain surface. Animals were sacrificed 12 days later. A zone of necrosis was visible macroscopically and confirmed with cresyl violet staining, and it included the ventral part of the hippocampus, entorhinal cortex, part of the amygdala, and the tail of the caudate. Some control and lesioned animals to be processed for immunohistochemistry received intraventricular (left side) injections of colchicine (20 μl; 6 μg/μl) 24 or 40 hr before sacrifice (6).

***In Situ* Hybridization.** Animals were decapitated, and the brains were removed, dissected, frozen, and sectioned in a cryostat (14 μm thick). Synthetic oligonucleotide probes complementary to rat galanin mRNA (nucleotides 230–277 and 152–199) (2) or rat ChAT mRNA (nucleotides 1818–1860) (15) were labeled at the 3′ end with deoxyadenosine [α-[^{35}S]thio]triphosphate (New England Nuclear) and terminal deoxynucleotidyltransferase (IBI). The sections were hybridized with 5×10^6 dpm/ml of the two galanin probes or 10^7 dpm/ml of the ChAT probe in a hybridization buffer containing 50% (vol/vol) formamide, and then washed with 1× SSC (0.15 M sodium chloride/0.015 M sodium citrate) at 55°C (16). Autoradiograms were generated by exposing the tissues to Amersham Hyperfilm βmax films (Amersham) or by dipping the sections in NTB_2 nuclear track emulsion (Kodak).

Immunohistochemistry. Brains were fixed, and frontal sections were cut (14 μm thick) at different levels with a cryostat and processed for the indirect immunofluorescence method, including absorption controls (6, 9). The sections were incubated with rabbit antiserum to synthetic galanin (1:400;

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Abbreviations: ChAT, choline acetyltransferase; 5-HT, 5-hydroxytryptamine (serotonin); IbA, ibotenic acid; -LI, -like immunoreactivity.

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Peninsula Laboratories), rinsed, and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit antibodies (1:40 or 1:80; Boehringer Mannheim). After photography, sections from the dorsal raphe nucleus were restained with guinea pig anti-5-HT antibodies (1:400) (17) as primary antibody followed by tetramethylrhodamine isothiocyanate-conjugated goat anti-guinea pig antiserum (1:20; Cappel Laboratories).

Analysis of the Results. Sections were examined in a Nikon Microphot-FX microscope equipped for epifluorescence and appropriate filters and with dark- and bright-field condensors. Galanin-LI- and galanin mRNA-positive cell profiles were counted in sections taken at regular intervals (one slice out of three) covering the whole extent of the medial septal nucleus and vertical diagonal band. Quantification of film autoradiograms was carried out by microdensitometry using a Macintosh IIx image-analysis and Image software program (courtesy of Wayne Rasband, National Institute of Mental Health, Washington, DC). The system was calibrated with the set of standards present in each film.

RESULTS

Decortication. *In situ* hybridization revealed numerous galanin mRNA-containing cell profiles in the dorsal raphe nucleus (Fig. 1 *a* and *d*). Four groups of labeled cells could be distinguished—a dorsomedian, a ventromedial, and two lateral groups forming bilateral wings. However, at rostral levels only a single midline group could be seen, and at caudal levels the ventromedial group disappeared. After bilateral decortication the levels of galanin mRNA markedly increased in the dorsal raphe complex (Fig. 1 *b* and *e*). Unilateral lesions resulted in a bilateral increase in galanin mRNA, which was more pronounced in the ipsilateral part of the nucleus (Fig. 1 *c* and *f*). The quantitative data are summarized in Fig. 2*a*. At the cellular level, the labeling was heterogeneous after decortication, and cells exhibiting high densities of grains were intermingled with more weakly labeled cells (Fig. 1 *b*, *c*, *e*, and *f*). In contrast, in control animals dorsal raphe cells were homogeneously labeled (Fig. 1 *a* and *d*).

In decorticated rats galanin mRNA signal was also seen in cell profiles in the ipsilateral dorsal and ventral thalamic

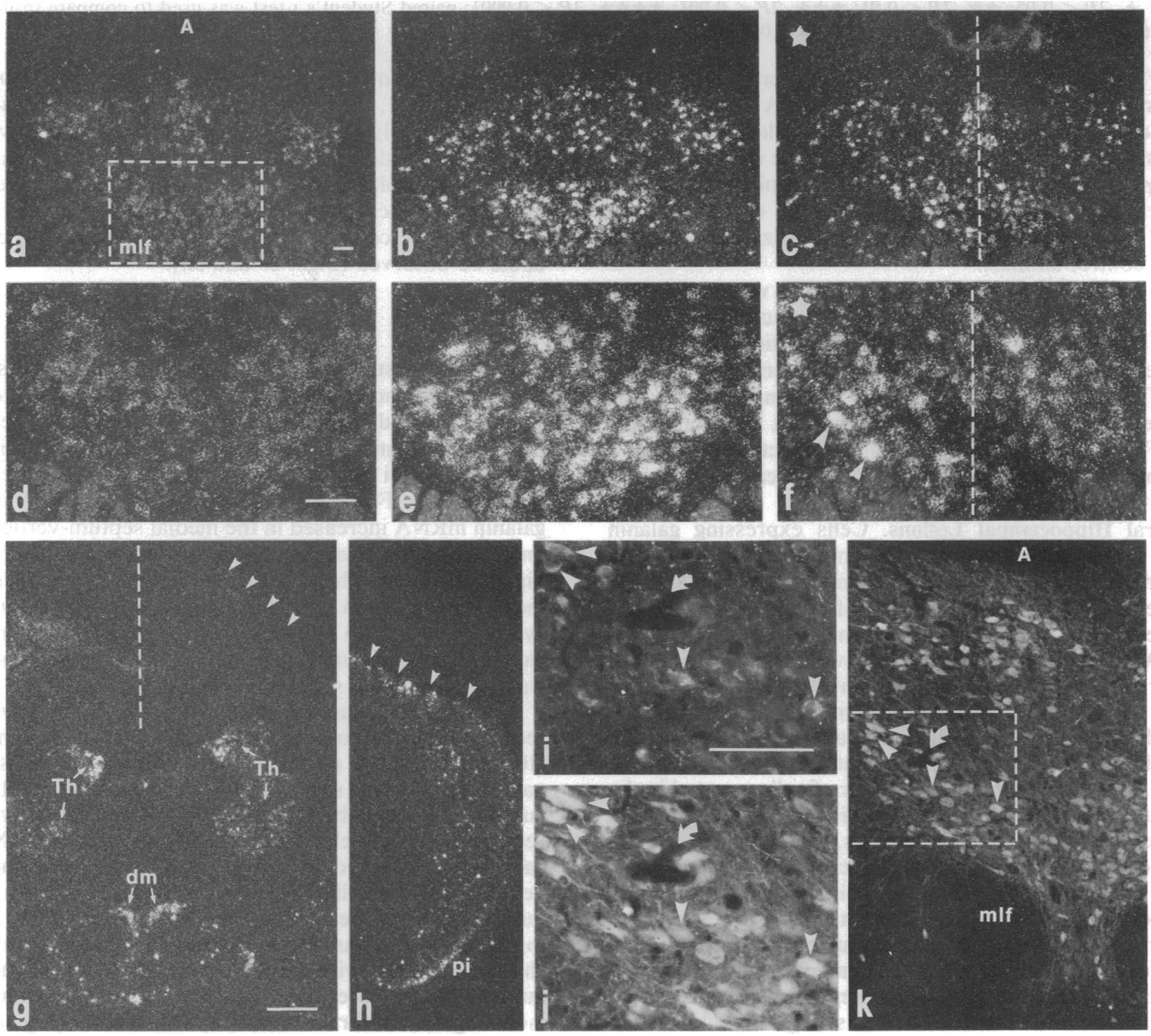

FIG. 1. (*a–f*) Dark-field autoradiographs of emulsion-dipped sections showing galanin mRNA labeling in the dorsal raphe nucleus [and detail of its ventromedial part (*d–f*)] of a control rat (*a* and *d*) and after bilateral (*b* and *e*) or unilateral (*c* and *f*) decortication. Box in *a* outlines area shown in *d*. The signal is stronger in lesioned animals. Note that after unilateral decortication galanin mRNA levels increase especially in the ipsilateral side (☆) (*f*). Arrowheads point to strongly labeled cells. (*g* and *h*) Film autoradiographs show galanin mRNA in some nuclei of the thalamus (Th) (*g*) and in the cortex (*h*), including the piriform cortex (pi), after bilateral decortication. Arrowheads point to the area of the lesion. dm, Dorsomedial hypothalamic nucleus. (*i–k*) Immunofluorescence micrographs show the same section with double-labeling of galanin- (*i*) and 5-HT-LI (*j* and *k*) in dorsal raphe nucleus cells after decortication. (*j*) Higher magnification of the boxed area in *k*. Arrowheads point to double-labeled cells. Curved arrows point to the same blood vessel (for orientation). A, aqueduct; mlf, medial longitudinal fasciculus. Dashed lines in *c*, *f*, and *g* indicate midline. [Bars = 100 μm (*a* = *b* = *c*; *d* = *e* = *f* = *k*; *i* = *j*) or 1 mm (*g* = *h*).]

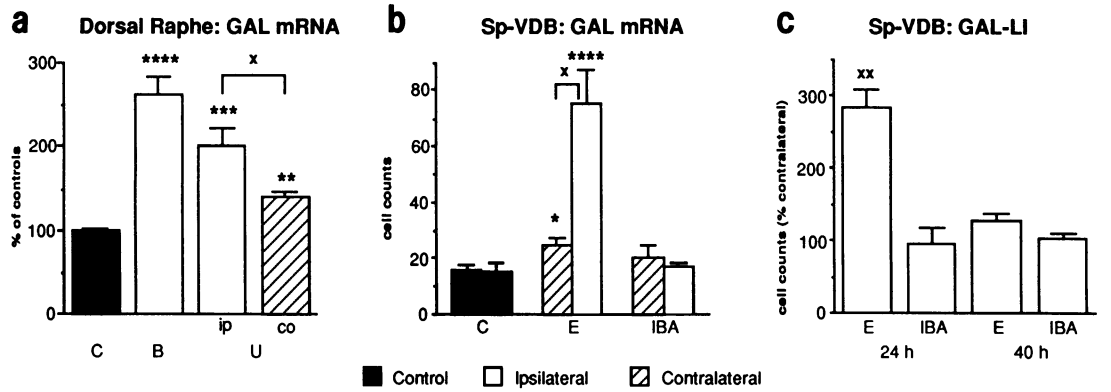

FIG. 2. (*a*) Levels of galanin mRNA (in % control values) measured in the dorsal raphe nucleus from control rats (C, $n = 8$) and rats with bilateral (B, $n = 6$) or unilateral (U, $n = 12$) decortication. ip, ipsilateral; co, contralateral side. (*b*) Number of galanin mRNA-containing cells counted in the septum-vertical diagonal band (Sp-VDB) in control rats (C, $n = 4$) and after lesions in the ventral hippocampus by electrocoagulation (E, $n = 4$) or IbA (IBA, $n = 4$). (*c*) Percentage of galanin immunoreactive cell counts in the ipsilateral versus the contralateral septum-vertical diagonal band (Sp-VDB) in animals lesioned in the ventral hippocampus by electrocoagulation (E) or IbA (IBA), after treatment with colchicine for 24 hr (E, $n = 4$; IbA, $n = 3$) or 40 hr (E, $n = 3$; IbA, $n = 2$). Unpaired t test was used to compare lesioned with control animals: ★, $2P < 0.05$; ★★, $2P < 0.01$; ★★★, $2P < 0.001$; ★★★★, $2P < 0.0001$; paired Student's t test was used to compare ipsilateral to contralateral side in lesioned animals: x, $2P < 0.05$; xx, $2P < 0.01$.

nuclear groups, and a few in the contralateral anterodorsal nucleus (Fig. 1*g*), whereas no galanin mRNA signal could be detected in these areas in normal rats. Similarly, small cells expressing galanin mRNA were found in the remaining parts of the ipsilateral cortex (e.g., cingulate and piriform regions) and were mainly concentrated in layer II of the piriform cortex (Fig. 1*h*). Labeled cells in these localizations were not seen in control animals.

With immunohistochemistry, galanin-LI was not detectable in cell bodies in the dorsal raphe nucleus in normal control rats or after unilateral decortication, but a few positive cell bodies could be seen after bilateral decortication (Fig. 1*i*). Restaining of the sections with antiserum against 5-HT revealed that galanin-LI was present in serotonergic cells (Fig. 1 *j* and *k*). Galanin immunoreactive cell bodies could not be detected in thalamic or cortical structures in normal or decorticated rats.

Ventral Hippocampal Lesions. Cells expressing galanin mRNA were found scattered throughout the medial septum and vertical diagonal band (maximum nine galanin-mRNA-positive cells per side and section). Most cells exhibited low grain densities. In adjacent sections, numerous cells expressing ChAT mRNA were seen with an overlapping distribution.

After unilateral electrocoagulation of the ventral hippocampal area the number of galanin mRNA-positive cells in the medial septum-vertical diagonal band was 3-fold higher in the ipsilateral compared with the contralateral side (Figs. 2*b* and 3*a*). In addition, the total number of cells counted in the contralateral nuclei was higher than in control rats. In adjacent sections from the same animals, there seemed to be a slight reduction in the number of cells expressing ChAT mRNA ipsilaterally to the lesion, coinciding with the localization of cells with increased galanin mRNA levels (cf. Fig. 3 *b* with *a*). In contrast, in animals injected with IbA in the same area of the hippocampus, the number of galanin mRNA-positive cell bodies in the medial septum-vertical diagonal band was not different from controls (Fig. 2*b*). No certain modifications of ChAT mRNA in cells in this area were noticed.

With immunohistochemistry, no galanin-LI could be detected in cell bodies without colchicine treatment. Twenty-four hours after injection of the drug, a few galanin-immunoreactive cells (up to 10 per side and section) could be seen in the control medial septum-vertical diagonal band, and numerous cell bodies (>20 per side and section) were stained 40 hr after colchicine injection. In animals with ventral hippocampus-entorhinal cortex lesions given colchicine 24 hr before sacrifice, the number of cell bodies showing galanin-LI was markedly higher in the ipsilateral compared with the contralateral side (Figs. 2*c* and 3 *c–f*). When animals were treated with colchicine for 40 hr, no side differences were noticed (Figs. 2*c* and 3*g*). After IbA lesions combined with 24 hr or 40 hr of colchicine treatment, no significant changes could be observed (Figs. 2*c* and 3*h*).

DISCUSSION

The present results provide evidence that the expression of galanin is enhanced after mechanical lesions within the brain. Thus, we found increases in galanin mRNA in the dorsal raphe nucleus and the appearance of galanin mRNA in some thalamic nuclei and remaining parts of the cortex after decortication. Furthermore, the number of cells containing galanin mRNA increased in the medial septum-vertical diagonal band after electrocoagulation lesions of the ventral hippocampus-entorhinal cortex. At least in some cases, these increases in galanin mRNA appeared to result in enhanced synthesis of galanin (see below). In contrast, the number of galanin-mRNA-positive cells was not modified, when IbA was injected in the same area of the hippocampus.

Increases in galanin mRNA and galanin-LI have been recently reported to occur in dorsal root ganglion neurons (10, 11) and in the magnocellular secretory neurons of the paraventricular and supraoptic hypothalamic nuclei (12) after cutting their axons by sciatic nerve transection or hypophysectomy, respectively. The increases in galanin mRNA and galanin-LI observed in dorsal raphe and basal forebrain neurons are most likely related to the destruction of the terminal fields of these neurons as well, because they project to the lesioned areas. Thus, galanin-LI has been previously shown to coexist with 5-HT in neurons of the dorsal raphe nucleus (9) that are known to project to forebrain areas, including the cerebral cortex (18, 19). Similarly, galanin-LI has been found in cholinergic cell bodies in the medial septum and diagonal band (8), which project to the hippocampal formation (20–22), and retrograde tracing studies have shown that neurons in these nuclei containing both ChAT and galanin-LI project to the hippocampus (8, 23). Furthermore, neurotoxic lesions of the septum-diagonal band decrease galanin-immunoreactive fibers in the hippocampal formation (24). Similarly, direct damage of axonal processes of cortical neurons and thalamic neurons in the dorsal and ventral

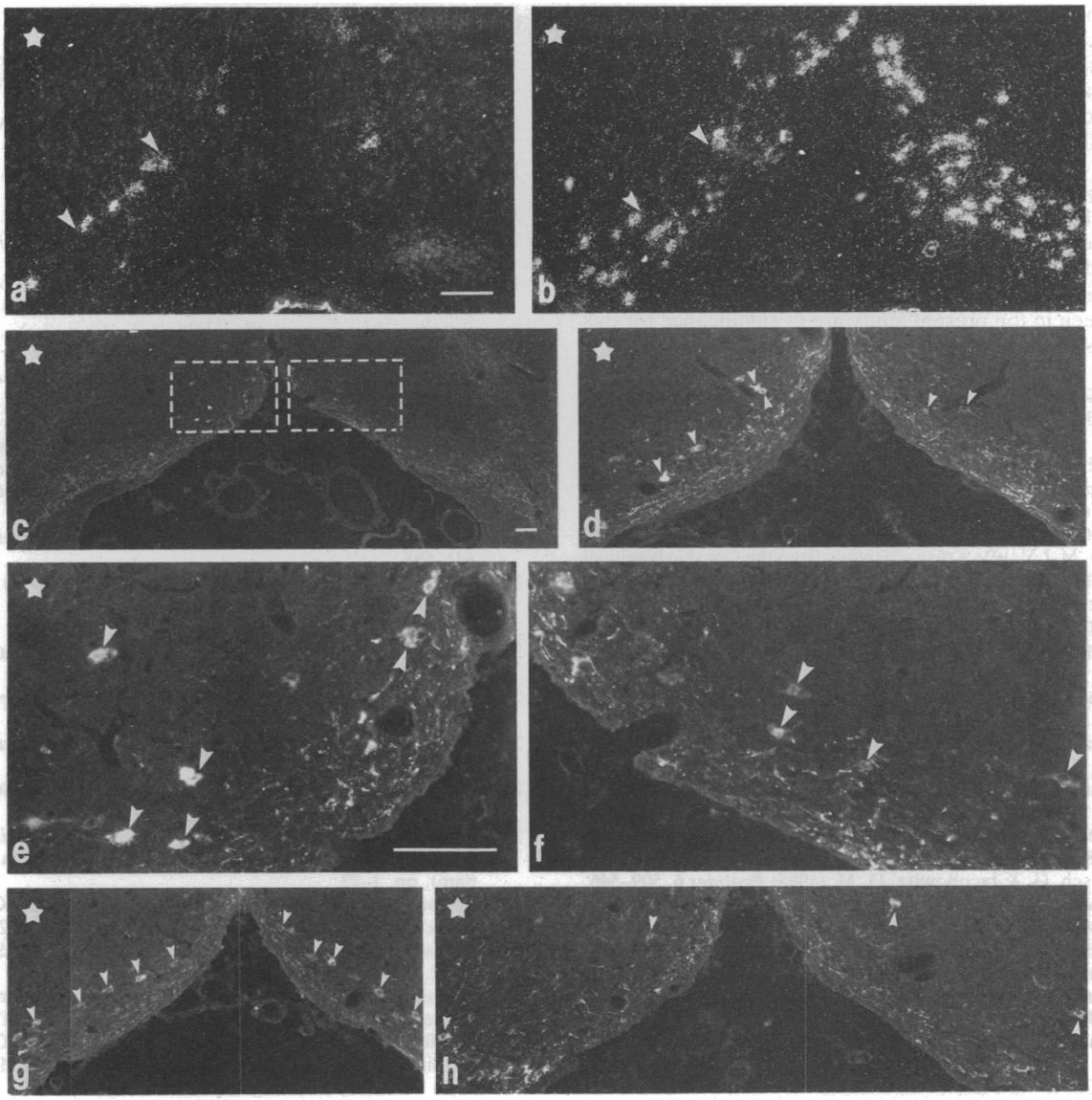

FIG. 3. (*a* and *b*) Dark-field autoradiographs showing galanin mRNA (*a*) and ChAT mRNA (*b*) in the vertical diagonal band after unilateral electrocoagulation of the ventral hippocampus. ☆, Lesioned side. Arrowheads delimit an area with increased galanin mRNA-positive cells and loss of ChAT mRNA ipsilaterally to the lesion. (*c–h*) Immunofluorescence micrographs showing galanin-LI in cell bodies (arrowheads) of the vertical diagonal band in animals lesioned in the ventral hippocampus by electrocoagulation (*c–g*) or IbA (*h*) and treated with colchicine for 24 hr (*c–f* and *h*) or 40 hr (*g*). (*e* and *f*) Higher magnifications of boxed areas in *c*. Arrowheads point to galanin-positive cells. Note the increase in galanin-LI in cells on the lesioned side (☆) after 24-hr colchicine (*c–e*), whereas no side difference is seen after 40-hr treatment (*g*). Only single weakly labeled cells without side difference are seen 24 hr after colchicine in IbA-treated rats (*h*). [Bars = 100 μm (*a* = *b* = *d* = *g* = *h*; *c*; *e* = *f*).]

nuclear groups is probably the cause of the induction of galanin mRNA expression, because these neurons are known to send projections, respectively, within or to the neocortex (see ref. 25). Finally, the fact that the destruction of target cells with IbA, which is supposed to spare axon terminals (26), does not change galanin mRNA levels, further supports the idea that a direct lesion of the axons is needed to increase galanin mRNA levels.

The present results are interesting in relation to a recent study by Sofroniew *et al.* (27) showing that transection of the fimbria causes a marked loss of ChAT-LI in basal forebrain neurons, whereas chemical destruction of the entire hippocampal formation by the excitotoxin *N*-methyl-D-aspartic acid (NMDA) causes no apparent changes of the basal forebrain cholinergic neurons, suggesting that these neurons are not dependent on hippocampal target neurons for their survival.

The present study also shows that not only galanin mRNA levels, but also galanin peptide, are affected by the mechanical lesions but are not affected by IbA. With regard to the dorsal raphe nucleus no galanin-positive cells were seen in controls, but after bilateral cortical lesions weakly immunoreactive cells were found, suggesting that increases in galanin mRNA had been translated into peptide. In the basal forebrain a more complex situation was encountered. Galanin-LI could not be seen either in normal rats or after lesion alone, but only after colchicine treatment. Thus, 24 hr after administration of this drug a marked increase in galanin-positive cells was observed on the side of electrocoagulation, and this correlated well with the increase in the number of galanin mRNA-positive cells (both up 300%). However, 40 hr after colchicine treatment no significant side differences could be seen, although the number of cells was markedly increased but in both sides. In fact, there were three to four times more

galanin-positive cells as compared with 24 hr after colchicine treatment. This is in all probability because colchicine by itself, as shown in our previous study (13), at this long time interval markedly upregulates galanin synthesis, possibly due to an effect on a retrogradely transported trophic factor. It appears, therefore, that the results of long-term colchicine treatment can mask the effects of the hippocampal electrocoagulation.

It is well known that cholinergic basal forebrain neurons are degenerated in Alzheimer disease (28), and it has been speculated that galanin synthesis may be upregulated during degeneration of these neurons (29). To what extent the changes seen in the present study are of significance for this hypothesis remains to be studied.

This work has been supported by the National Institutes of Mental Health (MH 43230-02), the Swedish Medical Research Council (04x-2887), Konung Gustav V:s och Drottning Victorias Stiftelse, Fredrik och Ingrid Thurings Stiftelse and Marianne och Marcus Wallenbergs Stiftelse. R.C. was supported by grants from Comissió Interdepartmental de Recerca i Innovació Tecnològica (CIRIT) (Generalitat de Catalunya) and Swedish Medical Research Council (14v-08726). M.J.V. was a reseach fellow from the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina. The support of the Wenner-Gren Center Foundation is gratefully acknowledged. We thank Dr. M. Schalling for supplying the oligonucleotide probes.

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