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Immunohistochemical evidence for the coexistence of histidine decarboxylase-like and glutamate decarboxylase-like immunoreactivities in nerve cells of the magnocellular nucleus of the posterior hypothalamus of rats

(histamine/γ-aminobutyric acid)

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ABSTRACT **Immunohistochemical staining of alternate consecutive sections revealed numerous histidine decarboxylase (L-histidine carboxy-lyase, EC 4.1.1.22)-like immunoreactive neurons that also contained glutamate decarboxylase (L-glutamate 1-carboxy-lyase, EC 4.1.1.15)-like immunoreactive structures in the tuberal magnocellular nucleus, the caudal magnocellular nucleus, and the postmammillary caudal magnocellular nucleus of the posterior hypothalamus of rats. Furthermore, in immunohistochemical double-staining procedures, almost all neurons in the magnocellular nuclei had both histidine decarboxylase-like and glutamate decarboxylase-like immunoreactivities. These results suggest the coexistence of histamine and γ-aminobutyric acid in single neurons in these nuclei.**

The possibility that histamine acts as a neurotransmitter or neuromodulator in mammalian brain has been suggested by neurochemical, neuropharmacological, and neurophysiological studies (1–3). Only recently, morphological evidence has been provided for a histaminergic neuron system: we showed by an immunohistochemical method with histidine decarboxylase (HDCase, L-histidine carboxy-lyase, EC 4.1.1.22), a histamine-synthesizing enzyme, as a marker that the tuberal magnocellular nucleus (TM), caudal magnocellular nucleus (CM), and postmammillary caudal magnocellular nucleus (PCM) are major sites in rat brain for formation of histamine (4–6). We also found that these neurons with HDCase-like immunoreactivity (Ir) had monosynaptic projections to the cerebral cortex (6).

At the same time, Vincent *et al.* (7) reported the existence of neurons with glutamate decarboxylase [GDCase, γ-aminobutyric acid (GABA)-synthesizing enzyme; L-glutamate 1-carboxy-lyase, EC 4.1.1.15]-like Ir in the TM, CM, and PCM. They found that these cells with GDCase-like Ir, similar to those with HDCase-like Ir, had projections to the cerebral cortex (8). These findings suggested that structures with HDCase- and GDCase-like Ir exist in single neurons in the TM, CM, and PCM, and thus these neurons have the capacity to synthesize histamine and GABA. To examine this possibility directly, we first used the procedure of immunohistochemical staining of alternate consecutive sections with anti-HDCase antiserum and anti-GDCase antiserum. To investigate the coexistence of these structures in detail, we developed an immunohistochemical double-staining technique to detect HDCase- and GDCase-like Ir separately in the same sections under a fluorescence microscope equipped with an appropriate filter mirror system.

MATERIALS AND METHODS

HDCase Antibody. HDCase was purified from fetal rat liver as described (9). The procedures used resulted in 1500-fold purification of HDCase, and the final preparation gave a single band of protein on 7.5% polyacrylamide gel electrophoresis. Antiserum to purified HDCase was produced in rabbit (4, 9). The HDCase antiserum was characterized by measurement of enzyme inhibition and by the Ouchterlony double diffusion test (4, 9). The specificity of this antibody was also evaluated by an absorption test. Anti-HDCase antiserum pretreated with excess HDCase served as control absorbed serum. Since no immunostaining was observed with the control absorbed serum, the antibody was concluded to stain specifically.

GDCase Antibody. Anti-GDCase antiserum S3 (fourth "bleed") was raised in sheep and characterized by enzyme precipitation and immunoelectrophoresis, as described in detail elsewhere (10). In short, GDCase-antiserum S3 precipitated 85% of the GDCase activity in the supernatant of a brain homogenate and retarded the mobility of one antigen in brain homogenate in crossed immunoelectrophoresis with intermediate gel. The immunohistochemical properties have been demonstrated in the cerebellar cortex (11) and other brain areas.

Immunohistochemical Studies of Alternate Consecutive Sections. Male albino rats (Wistar; Keary, Osaka, Japan) weighing ≈120 g were used. Colchicine (80 μg in 10 μl of saline) was injected into the third ventricle of animals under sodium pentobarbital anesthesia (10 mg/kg, i.p.). Twenty-four hours later, the animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused intracardially with 50 ml of ice-cold saline followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain was removed, immersed in the same fixative overnight at 4°C, and then rinsed for 48 hr in 0.1 M phosphate buffer (pH 7.4) containing 30% (vol/vol) sucrose. Serial frozen brain sections 5 μm thick were cut on a cryostat. Alternate sections were processed by the peroxidase-antiperoxidase method of Sternberger *et al.* (12) for demonstrating structures with HDCase-

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Abbreviations: HDCase, histidine decarboxylase; GDCase, glutamate decarboxylase; Ir, immunoreactivity; GABA, γ-aminobutyric acid; TM, tuberal magnocellular nucleus; CM, caudal magnocellular nucleus; PCM, postmammillary caudal magnocellular nucleus; FITC, fluorescein isothiocyanate.

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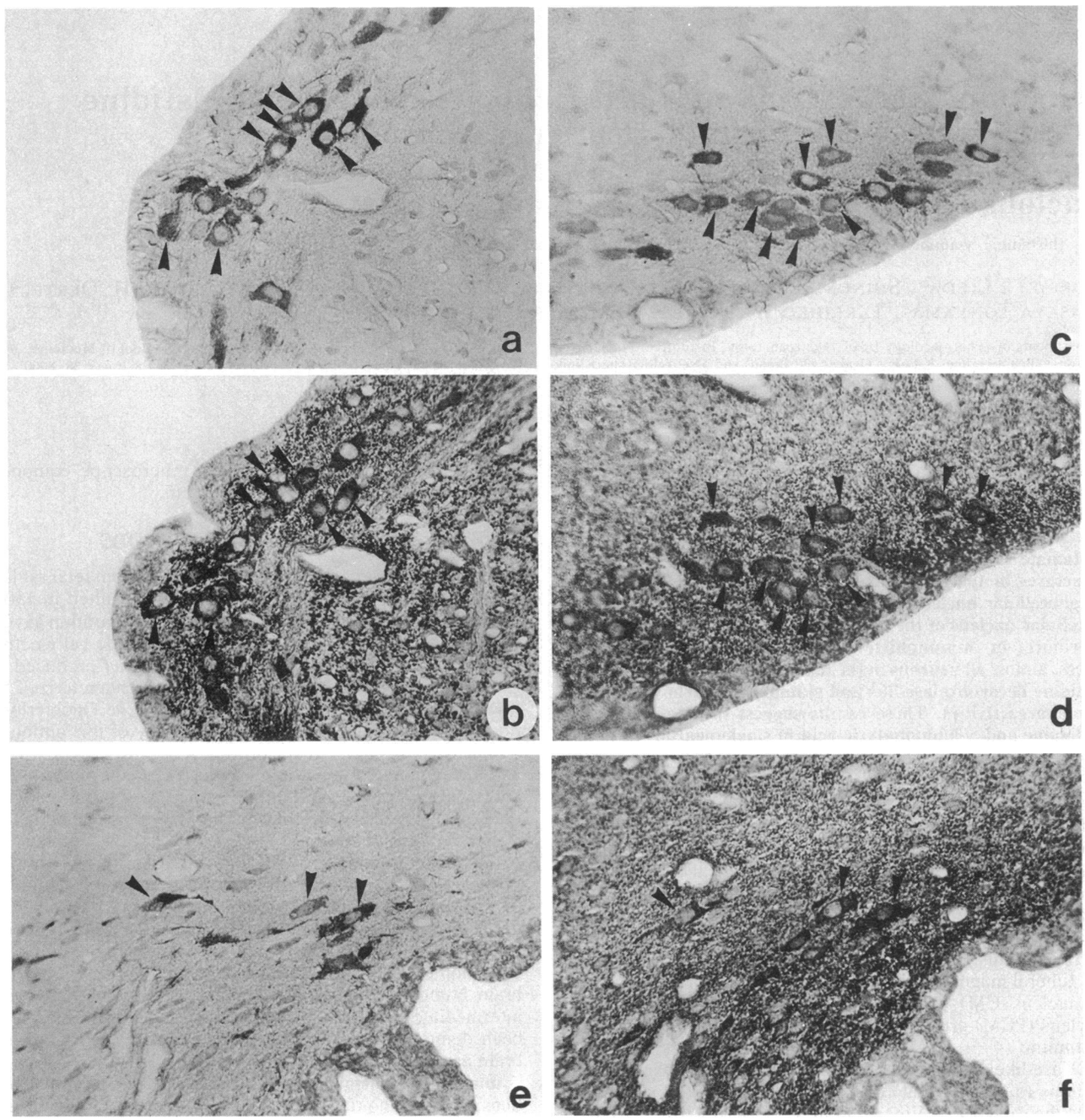

FIG. 1. Two serial frontal sections showing HDCase-like Ir neurons containing GDCase structures in the PCM, CM, and TM in colchicine-treated rats. Identical neurons in the two sections are indicated by arrowheads. HDCase-like Ir structures in the PCM (*a*), CM (*c*), and TM (*e*). GDCase-like Ir structures in the PCM (*b*), CM (*d*), and TM (*f*). (×240.)

or GDCase-like Ir. The first set of sections was sequentially incubated with anti-HDCase antiserum (1:2000, final dilution) in saline phosphate buffer (P_i/NaCl) overnight at 15°C, with goat anti-rabbit IgG (Boehringer, F.R.G.; 1:40, final dilution in P_i/NaCl) for 4 hr at 15°C and with rabbit peroxidase-antiperoxidase complex (DAKO, Glostrup, Denmark; 1:100, final dilution in P_i/NaCl) for 4 hr at 15°C. Each step was followed by several washes with P_i/NaCl. Finally, the sections were incubated for 20 min in a solution of 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) dissolved in 0.05 M Tris·HCl buffer (pH 7.4) and then for 5 min in the same solution containing 0.01% hydrogen peroxide.

The alternate sections were treated by the same procedures, but with the sequence of anti-GDCase antiserum (1:3000 final dilution in P_i/NaCl) as the primary antiserum, goat anti-sheep IgG (Miles; 1:20, final dilution in P_i/NaCl) and sheep peroxidase-antiperoxidase complex (Cappel Laboratories, Cochranville, PA; 1:50, final dilution in P_i/NaCl). Finally, the specimens were washed, dehydrated, mounted in balsam, and examined by light microscopy.

Immunohistochemical Double-Staining Technique. The procedure for obtaining specimens of rat brain was as described above. Frozen sections (20 μm thick) of the fixed brain were cut on a cryostat. They were first incubated in a mixture of rabbit anti-HDCase antiserum (1:1000, final dilution) and sheep anti-GDCase antiserum (1:3000, final dilution) in P_i/NaCl overnight at 15°C. Then they were washed with P_i/NaCl and incubated with a mixture of Texas Red-conjugated donkey anti-rabbit IgG (Amersham Japan, Tokyo, Japan; 1:100, final dilution) and a specific population of

fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat IgG (Nordic, Tilburg, Netherlands; 1:100, final dilution) in P_i/NaCl for 12 hr at 15°C. The sections were then rinsed with P_i/NaCl, mounted in P_i/NaCl/glycerine mixture (1:1), and examined by fluorescence microscopy. HDCase-like Ir structures showed red fluorescence with Texas Red when examined with a G dichroic mirror filter illumination system (Nikon), whereas GDCase-like Ir elements in the same sections showed weak green FITC fluorescence under B system illumination.

The techniques used depended on specific differences in the two primary antisera and the absence of cross-reactivities of the primary and secondary antisera. The absence of cross-reactivities was demonstrated by three lines of evidence. (*i*) Evidence for absence of cross-reactivity of anti-HDCase antibody with GDCase and of anti-GDCase antibody with HDCase: (*a*) In the cerebellum there were numerous structures with GDCase-like Ir, but few with HDCase-like Ir. (*b*) In the stomach, the basal granular cells had HDCase- but not GDCase-like Ir. (*ii*) Evidence for the absence of cross-reactivity of donkey anti-rabbit IgG with sheep IgG (anti-GDCase antiserum), and of donkey anti-goat IgG with rabbit IgG (anti-HDCase antiserum): (*a*) No red fluorescence was seen in the cerebellum after incubation with anti-GDCase followed by Texas Red-conjugated donkey anti-rabbit IgG. (*b*) We used a specific population of donkey anti-goat IgG. As commercial donkey anti-goat IgG (Amersham Japan, Tokyo, Japan) showed weak cross-reactivity with rabbit IgG (anti-HDCase antiserum) immunohistochemically, we eliminated this cross-reactivity by absorption of commercial donkey anti-goat IgG on an immunoaffinity column (Affi-Gel 10; Bio-Rad) coupled with rabbit IgG (Cappel Laboratories). The specificity of the absorbed antibody was tested immunohistochemically. No green fluorescence was seen in the TM, CM, and PCM or stomach after incubation with anti-HDCase followed by the FITC-conjugated absorbed donkey anti-goat IgG. (*iii*) There was no immunohistochemical cross-reactivity between donkey anti-rabbit IgG and donkey anti-goat IgG.

The nomenclature used was that of Bleier *et al.* (13).

RESULTS

On immunohistochemical staining of alternate consecutive serial sections, we detected numerous neurons that contained HDCase- and GDCase-like Ir. Fig. 1 shows a pair of serial sections of the PCM. The upper section (Fig. 1*a*) was stained for structures with HDCase-like Ir and the lower section (Fig. 1*b*) for profiles with GDCase-like Ir. Comparison of these sections showed that the neurons, indicated by arrowheads, were identical and contained both HDCase- and GDCase-like Ir. In addition, many neurons immunoreactive for HDCase in the CM (Fig. 1 *c* and *d*) and TM (Fig. 1 *e* and *f*) were also shown to exhibit GDCase-like Ir.

To investigate the coexistence of HDCase- and GDCase-like Ir in detail, we used the immunohistochemical double-staining technique. As shown in Fig. 2, almost all neurons in the CM exhibited both HDCase- and GDCase-like Ir. In regions outside the TM, CM, and PCM, however, such as in the region between the CM and the recessus mammillaris of the third ventricle (Fig. 3), only a few neurons with GDCase-like Ir were detected, and these were negative for HDCase-like Ir. In other areas of the brain, the distributions of structures with HDCase- and GDCase-like Ir were also quite different. For example, in the cerebellum, there were numerous

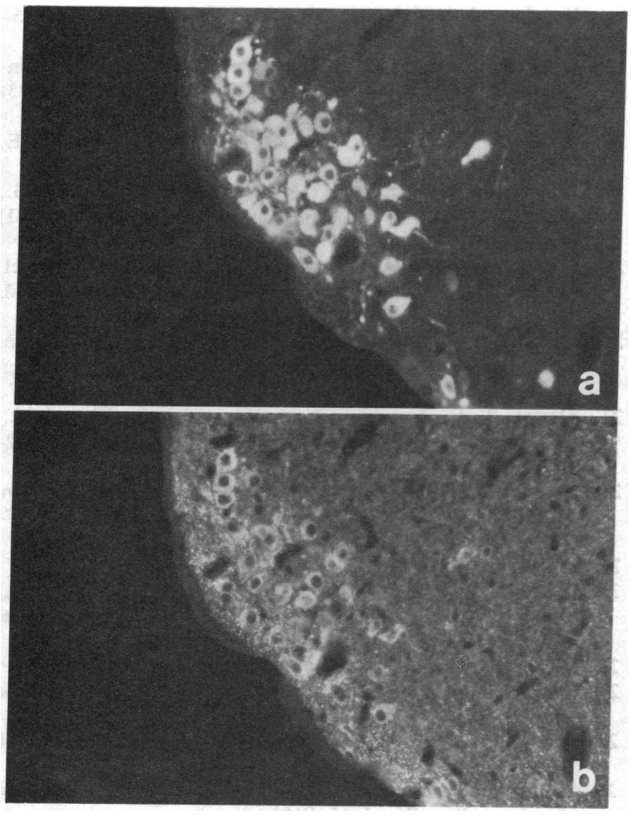

FIG. 2. Double immunofluorescent photomicrographs of the same section of the caudal magnocellular nucleus in a colchicine-treated rat. (*a*) HDCase-like Ir neurons showed red fluorescence with Texas Red, and (*b*) GDCase-like Ir neurons showed weak green FITC fluorescence. Note that almost all neurons in this nucleus showed both HDCase- and GDCase-like Ir. (×150.)

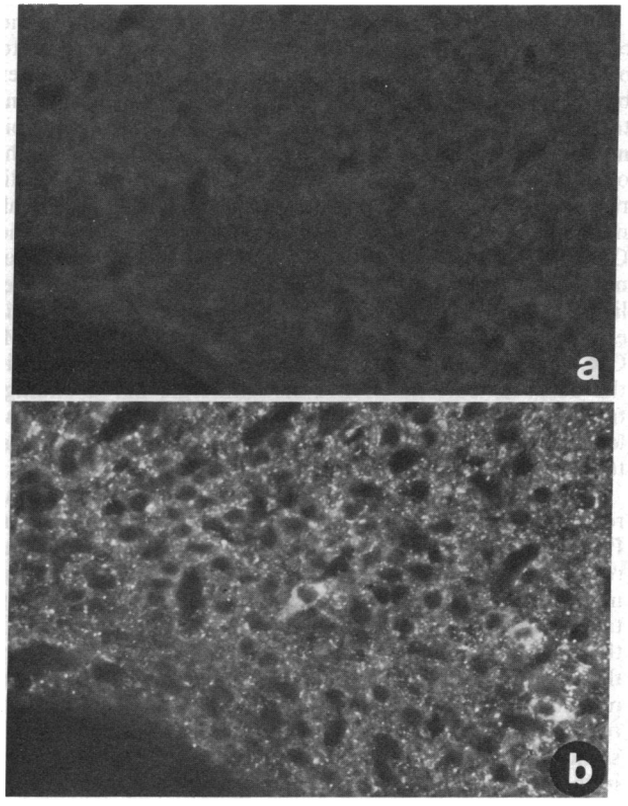

FIG. 3. Double immunofluorescent photomicrographs of the same section of the arcuate nucleus in a colchicine-treated rat. Three neurons with GDCase-like Ir identified by their weak green FITC fluorescence (*b*) did not show red fluorescence with Texas Red for HDCase-like Ir (*a*). (×300.)

structures with GDCase- but few with HDCase-like Ir. The latter findings substantiate the conclusions from control experiments that primary and second antisera did not cross-react and that a filter system separated the two fluorescence markers effectively.

Based on these data, it is concluded that the same neurons in the TM, CM, and PCM contain both HDCase- and GDCase-like Ir.

DISCUSSION

The coexistence of neurotransmitters and neuromodulator substances in the same neurons in several regions in the brain has been reported (14–16). The present study demonstrated that TM, CM, and PCM neurons contain both HDCase- and GDCase-like Ir. This strongly suggests the coexistence of histamine and GABA in single neurons in the TM, CM, and PCM.

The coexistence of monoamine and GABA has not been observed previously, except in a small population of cells in the dorsal raphe nuclei, which have been reported to contain both serotonin- and GDCase-like Ir (17). Dopamine and GABA were clearly present in different classes of neurons in the zona incerta/A13, the substantia nigra, the reticular formation, and the olfactory bulb in rat brain (18). In addition, various neuropeptides have been found in the same GABAergic neurons in several regions: some Purkinje cells contained both motilin- and GDCase-like Ir (19), opioid peptide-like Ir was present in a subclass of medium-sized GDCase-like Ir striatal neurons (20), cholecystokinin-like Ir has been demonstrated in a subclass of GDCase-like Ir non-pyramidal hippocampal neurons (21), and somatostatin-like Ir was found in neurons of the feline thalamic reticular nucleus (22). In addition, β-cells in the pancreas containing insulin- and GDCase-like Ir have been reported (23).

Many GABAergic somata and fibers were present in the cerebral cortex (24, 25). Most cortical GABA is thought to be of intrinsic origin, because sectioning of the white matter below the cortex did not reduce the GABA and GDCase contents of the cortex (26, 27). However, we found by a combination of immunohistochemical and retrograde tracer methods that HDCase-like Ir cells in the TM, CM, and PCM directly innervated the cerebral cortex (6). Since almost all neurons in these nuclei contained both HDCase- and GDCase-like Ir, it seems likely that the HDCase-like immunoreactive projections to the cortex also contain GDCase-like Ir. Thus, the cerebral cortex may receive GABAergic efferents from GABA histaminergic neurons within the TM, CM, and PCM. The existence of two GABAergic systems in the cerebral cortex, GABA histaminergic nerve endings from projection neurons in the posterior hypothalamus and local circuit GABAergic neurons, suggests that the two systems have discrete functional roles.

Recently, Nagy *et al.* demonstrated the existence of neurons with adenosine deaminase-like Ir in the TM, CM, and PCM which project into the cerebral cortex (28). Single neurons in these nuclei may contain HDCase-, adenosine deaminase-, and GDCase-like Ir, suggesting the coexistence of histamine, GABA, and adenosine as neurotransmitters. Furthermore, neurons in the TM, CM, and PCM also show thyroid stimulating hormone-releasing hormone-like immunoreactive structures (29). The possibility of the coexistence of HDCase, GDCase, adenosine deaminase, and thyroid stimulating hormone-releasing hormone in the same neurons in these magnocellular nuclei remains to be examined.

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