 DISTRIBUTION OF NEURONAL CELL BODIES AND AXONS CONTAINING GLICENTIN-LIKE IMMUNOREACTIVITY IN THE RAT BRAIN

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Neuronal structures containing glicentin- or pancreatic glucagon-like immunoreactivity were studied in the rat brain by peroxidase anti-peroxidase immunohistochemistry. With anti-glicentin 49-69 serum R-4804, which recognized gut type glucagon but did not react with pancreatic type glucagon, immunoreactive nerve fibers and terminals were distributed in several brain regions including the hypothalamus. No immunoreactive neuronal soma was found in intact rats. Intraventricular injection of colchicine successfully revealed cell bodies with glicentin-like immunoreactivity in the lower medulla oblongata. These neuronal somata, though few in number, were mainly localized in a region between the nucleus tractus solitarius and nucleus dorsalis nervi vagi. Ventrolaterally to this region, some positive cells were also scattered up to the nucleus reticularis lateralis. The location of these glicentin-like immunoreactive cells closely resembled that of catecholamine cell groups A1 and A2. Our results strongly suggest that the rat brain contains gut type but not pancreatic type glucagon, and here a map of glicentin-like immunoreactive neuronal structures was given in detail.

Recent advances in peptide research indicate that many bioactive peptides including hormones are present incidentally in both the central nervous system (CNS) and peripheral organs. In the case of the glucagon family, pancreatic type glucagon, a well-known hormone localized in islets of Langerhans, and also another types of glucagon have been demonstrated to be present in extrapancreatic digestive organs such as the oxyntic gland, intestinal mucosa and salivary gland. The extrapancreatic glucagon has been termed "gut type glucagon" or "entero-glucagon".

More recently, the results of immunohistochemical studies have indicated that the CNS contains either pancreatic or gut type glucagon or both. For example, Loren et al. (10) reported that gut type glucagon were detected in nerve fibers of the rat brain. Their conclusion was, however, deduced from results obtained by using four different antisera; two of which were specific for pancreatic type glucagon, while the other two crossreacted with gut type glucagon. Therefore, no direct proof, such as using antiserum specific for gut type glucagon, was given. Dorn's group employed antiserum reacting with both pancreatic and gut type glucagon, and suggested that glucagon-like immunoreactivity was present in the CNS of rodents (4) and humans (5, 6). However, precise knowledge concerning the chemical
composition of the ‘glucagon-like immunoreactivity’ was unclear. Tager et al. (15), by using pancreatic type glucagon antisera which possessed a little cross-reaction with enteroglucagon, also suggested the presence of glucagon-containing peptides which displayed some properties of gut type glucagon in neuronal cells of the rat hypothalamus. In these experiments, however, it appeared difficult to identify the chemical structure of glucagon-like immunoreactivity, mainly due to the lack of the use of a monospecific antibody.

On the other hand, Thim et al. (16) purified a peptide, termed glicentin, from extracts of the gastrointestinal tract. Similarity in chemical structure indicated that the peptide was a member of the glucagon family, because among the 69 amino acid residues, the entire amino acid sequence of pancreatic type glucagon was included. Thus glicentin has been conceived of as one of the gut type glucagon-related peptides. The N-terminus of the glucagon molecule in glicentin was connected to a peptide with 32 amino acid residues (a N-terminal region), and the C-terminus to an octapeptide (a C-terminal region). Production of glicentin N-terminal (12) or C-terminal (19) specific antibodies, reacting with glicentin 10–30 or glicentin 49–69, respectively, has permitted the differentiation of glicentin from pancreatic type glucagon. Triepel et al. (18) were the first who applied such a region specific antibody, glicentin N-terminal specific antiserum R-64 developed by Ravazzolla et al. (12), to an immunohistochemical study of gut type glucagon-related peptides in the guinea pig brain.

In the present study, we have used another region specific antibody, glicentin C-terminal specific antiserum R-4804 developed by Yanaihara and coworkers (19), for the investigation of neuronal structures with glicentin-like immunoreactivity in the rat brain. Furthermore, the possible existence of pancreatic type glucagon in the rat CNS has been examined also immunohistochemically by using an antiserum GA-10 which recognizes both pancreatic and gut type glucagon, or an antiserum OAL-123 which is specific only for pancreatic type glucagon. Our results strongly suggest that the rat brain contains gut type but not pancreatic type glucagon, and here a map of glicentin-like immunoreactive neuronal structures is given in detail.

MATERIALS AND METHODS

Ten male Wistar rats weighing 200–250 g were used. Five animals received colchicine (150 µg in 20 µl saline) into the lateral cerebral ventricle 24 hr prior to sacrifice. Under anesthesia with urethane (0.8 g/kg) plus a-chlorarose (0.06 g/kg), the animals were perfused through the left ventricle first with 80 ml of 0.01 M phosphate-buffered saline and then with 300 ml of a fixative containing 4% paraformaldehyde, 0.5% glutaraldehyde and 0.2% picric acid buffered with 0.1 M phosphate buffer (pH 7.4). The fixed brains were removed from the skull and cut into coronal blocks of 5–6 mm thickness. The tissue blocks were postfixed for 2 days in the glutaraldehyde free fixative at 4°C and placed for at least 48 hr in phosphate-buffered 15% sucrose at 4°C. The blocks were frozen by CO₂ and sectioned at 20 µm in a cryostat. The sections were stored for 4 days in phosphate-buffered saline containing 0.3% Triton X-100 (0.9% NaCl in 0.1 M phosphate buffer, pH 7.4; PBST). Serial sections of 200 µm intervals were used for peroxidase
anti-peroxidase immunocytochemistry. The sections were always treated as free floating through the colorization step. All sera were diluted with PBST, and sections were washed also with PBST after each incubation. One of three antisera was used as a primary antibody; glicentin C-terminal 49–69 specific antiserum R-4804 (19) for detecting glicentin-like immunoreactivity, or glucagon C-terminal 19–29 specific antiserum OAL-123 (20) for detecting pancreatic glucagon-like immunoreactivity, or glucagon N-terminal 11–15 specific antiserum GA-10 (7) for detecting both pancreatic and gut type glucagon-like immunoreactivities. Appropriate dilutions of the antisera were 1 : 20,000 for R-4804, 1 : 4,000 for OAL-123, and 1 : 4,000 for GA-10. The procedure for staining was as follows.

1) Incubate sections for 3 days with a primary antiserum at 4°C.
2) Incubate for 6 hr with goat anti-rabbit IgG (MBL, Japan) diluted 1 : 3,000 at 4°C.
3) Incubate for 2 hr with peroxidase anti-peroxidase complex (MBL, Japan) diluted 1 : 2,000 at room temperature.
4) Colorize sections by reacting for 20 min with 0.05 M Tris-HCl buffer (pH 7.6) containing 0.02% 3,3'-diaminobenzidine, 0.005% H₂O₂ and 0.3% nickel ammonium sulfate.
5) Terminate the reaction by replacing sections in 10 mM phosphate buffer (pH 7.4).
6) Mount the sections on chrome alum-coated glass slides, dehydrate and cover-slip for examination.

Absorption tests for specificity were carried out as follows; 200 µg of synthetic glicentin 49–69 was added to 1 ml of diluted serum R-4804, or 10 µg of porcine glucagon (Novo) to 1 ml of diluted serum GA-10, at their working dilutions used for immunohistochemistry. The antigen-mixed antisera were incubated for 72 hr before staining.

RESULTS

Immunohistochemical controls for specificity, using normal (non-immune) rabbit sera or antigen-absorbed antisera, showed no positive staining. A further absorption control for immunohistochemistry was carried out by adding various concentrations of bovine serum albumin (0.1–100 µg) to 1 ml of diluted primary antisera. The results clearly indicated that the positive staining was not related to the carrier protein of haptenic immunogens. The use of either GA-10 or R-4804 serum gave specific immunoreactive staining, whereas no positive reaction was observed with the use of OAL-123 serum. Although the pattern of staining, in terms of both morphology and distribution of positively stained structures, appeared to be fundamentally identical with both GA-10 and R-4804 antisera, staining intensity observed with R-4804 was much stronger than that with GA-10. We describe here, therefore, the results obtained with R-4804 antiserum.

Glicentin C-terminal immunoreactive components were exclusively localized in neuronal elements, especially axons and terminals, while no immunoreactive cell body was found in pharmacologically unmanipulated animals. Intraventricular injection of colchicine, however, not only enhanced intensity of immunoreactive staining in nerve fibers and terminals but also revealed positive cell bodies. These
Figs. 1A–C. Micrographs of glicentin immunoreactive neuronal structures in the brain of rats. 
A and B. Positive cell bodies in a region between the nucleus tractus solitarius and nucleus 
dorsalis nervi vagi of a colchicine-treated rat. The level of B is 60 µm caudal to that of A. Smaller 
arrow heads indicate positive large varicosities of neuronal processes, while a larger marker indi-
cates positive reaction in an erythrocyte due to endogenous peroxidase activity. Bars=10 µm
C. Positive dot-like structures indicating nerve terminals and a few varicose nerve fibers distributed 
in the nucleus paraventricularis hypothalami of an intact rat. Bar=200 µm
Inset of C. Higher magnification of the triangulated area showing clear feature of an immuno-
reactive varicose fiber that is situated in a region adjacent to the dorsolateral shoulder of the paraventricular nucleus. Bar=20 µm
Fig. 2. A series of drawings of coronal sections through the brain of a colchicine-treated rat, illustrating the distribution of glicentin immunoreactive structures on the right side of the diagrams. Immunoreactive cell bodies seen only in the medulla oblongata are represented by filled circles, while positive nerve fibers and terminals are by small dots.
cells, though few in number, were situated in the lower levels of the medulla oblongata. They were mostly medium to large sized (15–23 µm in diameter) and had long ovoid perikarya (Figs. 1A, 1B). The location of the positive cell bodies was fairly limited in narrow regions: predominantly in a region between the tractus solitarius and nucleus dorsalis nervi vagi, and partly in a region adjacent to ventral and lateral borders of the nucleus reticularis lateralis (Fig. 2). It should be noted that the total number of these positive cells was rather small, since only a few positive cells were seen in each of several serial sections of 20 µm thickness.

Glicentin-like immunoreactive nerve fibers and terminals were distributed widely in various brain regions of both intact and colchicine-treated rats. Although the colchicine treatment increased the staining intensity which made for easier observation in detail, the treatment appeared not to affect the distribution pattern of positive nerve fibers and terminals. Therefore, the data obtained in colchicine-treated animals were represented schematically in Fig. 2. The hypothalamus was the most conspicuous region, because intensely stained dot-like structures suggesting nerve terminals were densely packed in certain nuclei. Several of these immunoreactive dots often formed a line implying that those were short varicose axons, and occasionally relatively long varicose axons were clearly visible even in an intact rat (Fig. 1C; inset). The hypothalamic nuclei possessing a high density of positive axonal components were the nucleus paraventricularis (Fig. 1C), nucleus ventromedialis and nucleus arcuatus. A moderate density of positive dot-like structures and varicose fibers were also seen in the nuclei septi lateralis et medialis, nucleus tractus diagonalis (Broca), nucleus periventricularis hypothalami, nucleus supraopticus, nucleus periventricularis thalami, area tegmentalis ventralis, periaqueductal gray matter at levels from the midbrain to the medulla, and raphe regions of the medulla (Fig. 2). As shown in Fig. 2, the positive axons and terminals in the amygdaloid complex did not show a particular pattern of localization in any subnuclei of the complex. Although the size of the immunoreactive dots or varicosities in the above regions was generally less than 3 µm in diameter, that of positive cell bodies in the medulla was mostly larger (5–6 µm in major axis) in colchicine-treated animals. Such a swelling of varicosity seemed to reflect that glicentin immunoreactive materials were accumulated, by the colchicine treatment, in the varicose segments of neuronal processes of the positive cell bodies. In fact, no swollen varicosity was seen in any medullary section of intact rats.

**DISCUSSION**

The present immunohistochemical study revealed no positive immunoreaction with pancreatic type glucagon and, instead, demonstrated the existence of a glicentin-like immunoreactivity in neuronal structures of the rat brain. Similar immunohistochemical results have been reported in brains of various mammals (4, 10, 15, 18) including human (5, 6). However, immunoreactivity assumed to be a pancreatic type glucagon has been shown in some neurons of the brain (2, 13, 17). This discrepancy is probably due to differences in specificity of the antisera used. Glicentin with 69 amino acid residues contains a whole pancreatic type glucagon sequence with 29 amino acid residues (9, 16). However, glicentin seldom crossreacts with an antibody against the C-terminus of pancreatic type glucagon such as
antiserum OAL-123. This is because its antibody recognition site is masked by an octapeptide extension, the C-terminus of glicentin, which is commonly shared by oxyntomodulin and proglucagon (1, 16). On the other hand, antiserum R-4804 mainly detects this C-terminal octapeptide extension, but does not recognize pancreatic type glucagon (19). All the above properties of antibody specificity strongly suggest that the C-terminal octapeptide is an important antibody recognition site for discriminating gut type glucagon from pancreatic type glucagon. Another way to discriminate these is, as Tripel et al. reported (18), to employ glicentin N-terminal specific antiserum. In fact, the pattern of distribution of the glicentin C-terminal specific immunoreactivity in the rat brain was substantially identical with that of N-terminal specific immunoreactivity in the guinea pig brain (18).

Although Loren et al. (10), using non-region-specific antisera, showed almost the same distribution as our results concerning positive nerve fibers in the rat brain, they did not report any positive cell bodies. This may not be surprising, since they used intact colchicine-untreated rats in which no positive cell body has been found in the present study either.

One noteworthy finding is that the distribution of neurons with glicentin C-terminal specific immunoreactivity closely resembles that of catecholaminergic neurons of A1 and A2 in the medulla oblongata (3). Our latest study has shown that the majority of glicentin-positive neurons contained catecholamine (unpublished data). It has been also observed that treatment with a MAO inhibitor markedly enhanced immunostaining for glicentin, while reserpine brought about a dramatic decrease. The changes were best observed in the nucleus paraventricularis of the hypothalamus. Catecholamine-containing cell bodies in A1 and A2 have been shown to project to the nucleus paraventricularis (14). Therefore it is suggested that glicentin coexists in catecholamine neurons which project their axons to the hypothalamus, especially the nucleus paraventricularis and nucleus supraopticus.

Physiological roles of glicentin in the CNS are unknown, though a few clues may be obtained. For instance, food deprivation raised glicentin contents in the rat hypothalamus (8). This implies an involvement of glicentin containing neurons in feeding behavior. Moreover, glucose responding neurons have been found in the nucleus tractus solitarius (11). The relationship between these glucose responding cells and glicentin-containing cells is noticeable. Evidently, basic knowledge concerning the role of glicentin, for example, as a neurotransmitter or neuromodulator is urgently required.

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