**GABA-T-POSITIVE NEURONS IN THE RAT
SUPRAOPTIC NUCLEUS AS REVEALED
BY A PHARMACO-HISTOCHEMICAL
METHOD WITH GABACULINE**

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The pharmacohistochemical method with gabaculine treatment for selectively detecting the enzyme 4-aminobutyrate: 2-oxoglutarate (EC. 2.6.1.19.: GABA-T) in neuronal perikarya was applied to the rat supraoptic nucleus (SO) to detect GABA-T-intensive, presumably GABAergic neurons in and around the SO, and if possible, to examine their relationship to neurosecretory neurons.

A significant number of medium-sized GABA-T-intensive neurons were detected in the dorsal part, while more numerous, small, moderately GABA-T-positive neurons were detected in the ventral part of the perinuclear zone just dorsal to the SO. Inside the SO, no GABA-T-intensive intrinsic neurons were detected, although a few small, mildly GABA-T-positive ones were detected in a few cases. No projections of these GABA-T-intensive neurons to the SO were detected. The unreactive cell bodies of some neurosecretory neurons located in the dorsal margin of the SO as well as in discrete groups scattered throughout the SO were surrounded by granular reaction products for GABA-T.

The results strongly suggest that the medium-sized GABA-T-intensive neurons in the perinuclear zone send their axons to the SO, where they richly divide into many branches which surround the cell bodies of some oxytocin and vasopressin cells with abundant GABA-T-positive axon terminals.

A Golgi study on the rat supraoptic nucleus (SO) identified intrinsic neurons, but it did not demonstrate the destinations of their cell processes (1). Quantitative electron microscopic studies (11, 28) assumed the presence of intranuclear richly arborizing axons, but failed to identify intrinsic neurons due to the paucity of these cells in the rat SO.

The presence of inhibitory interneurons has been proven by electrophysiology in the recurrent collateral system of the SO in the dog and cat (8), but not in the rat (7). However, monophasic specific osmonsensitive cells in the perinuclear zone around the SO have been classified into excitatory and inhibitory neurons in the unanesthetized monkey (3).

Biochemical studies suggest that 50% of glutamic acid decarboxylase (GAD) in the SO is in the intrinsic or the adjacent neuronal populations (14). An organ cultured study showed that cultured SO neurons of puppies were inhibited directly

by γ -aminobutyric acid (GABA) (21). However, immunocytochemical studies have not clearly shown the existence of inhibitory intrinsic neurons in the rat SO (19).

Recently, Nagai *et al.* (17) introduced a pharmacohistochemical method with gabaculine treatment for selectively detecting GABA-T-positive neuronal perikarya. They concluded that the distribution of GABA-T-intensive neurons in the rat brain was convincing evidence for the correspondence of GABA-T-intensive and GABA neurons (15, 17). Although they observed a few GABA-T-intensive neurons in the SO area, they did not describe them in detail (16).

The purpose of this study is to detect GABA-T-intensive, presumably GABAergic neurons to synthesize GABA-T rapidly in and around the SO, and if possible, to examine their relationship to neurosecretory neurons with the GABA-T method (17).

MATERIALS AND METHODS

Eleven female Wistar rats weighing from 180 to 200 g were used. The animals were divided into groups A ($n=5$) and B ($n=6$). The experiments were made under the two different conditions with regard to the dose of gabaculine (30 mg/kg in group A, 40 mg/kg in group B) and length of survival period (15 hr in group B, 24 hr in group A) after administration, but strictly according to the Nagai *et al.* (17) method. The cerebellum was used as a positive control. Negative control sections were incubated without the substrate GABA.

Nagai *et al.* (15, 17) used 50–75 mg/kg gabaculine to detect only GABA-T-intensive neurons for making a map of the distribution of these GABAergic neurons including very strongly positive striato-nigral complex in the rat brain. As the level of GAD in the SO is the lowest in discrete nuclei of the rat hypothalamus (24), the present study was programmed at first to detect GABA-T-containing neurons avoiding the glial staining, and next to limit the reaction to the GABA-T-intensive neurons in and around the SO, where the existence of GABAergic neurons remains unknown in the rat by immunocytochemistry (19). In the preliminary experiments, administration of either 30 or 40 mg/kg gabaculine gave identical results in the hypothalamic area of the rats without glial staining which had been allowed to live for 15 hours after administration. The cerebellum of group B, where the organization of GABA cells is particularly well understood (17), showed a similar reaction pattern as that described by Nagai *et al.* (15–17). Thus we selected the present experimental conditions as suitable for our purpose.

40 or 30 mg/kg gabaculine (Calbiochem) was injected to the V. saphena magna by surgical operation under slight ether anesthesia. Rats were killed 15 or 24 hours after administration of gabaculine by transcardial perfusion, first with 10 ml of 0.1 M phosphate-buffered saline solution (pH 7.4, ice-cold) for 60 to 90 sec., then with 150 ml of an ice-cold fixative solution (2% formaldehyde, 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4) for 10 to 13 min under artificial respiration, with anesthesia by intraperitoneal injection of 30 mg/kg pentobarbital. Prior to transcardial perfusion, 100 I.U. heparin had been injected to the left ventricle, the descending aorta ligated, and a cut made in the right atrium to allow outflow of the solutions. Perfusion pressure was obtained by a gravity feed system maintained at a height of 150 cms.

After perfusion, the rat brain was excised. The hypothalamus and cerebellum were removed, and they were cut into small cuboidal pieces of a width less than 5 mm. They were quickly placed in an ice-cold 0.1 M phosphate buffer solution (pH 7.4). 40 μ m Vibratome sections were cut serially with an Oxford Vibratome, and collected in the same buffer solution as described above. Groups of 2 or 3 sections were preincubated for 20 min at 37°C in 50 mM Tris-HCl buffer (pH 8.6) containing 5 mg/ml α -ketoglutarate (Sigma), 1 mg/ml nicotinamide adenine dinucleotide (NAD) (Sigma), 7.2 mg/ml NaCl, 1.02 mg/ml $MgCl_2$, 0.5 mg/ml malonate, and 0.05 mg/ml KCN. The substrate and dye were then added to the preincubation solution to give final concentrations of 5 mg/ml GABA (Sigma), 1 mg/ml nitro blue tetrazolium (Sigma), and 0.005 mg/ml phenazine methosulfate (Sigma). Free floating Vibratome sections were incubated for 45 min at 37°C in the dark. They were agitated by a rotator during incubation. The sections were then briefly rinsed twice in both 0.1 M phosphate buffer (pH 7.4, ice-cold, each 30 sec.) and distilled water (each, 30 sec.), dehydrated, and cover-slipped. Observations were made with a light microscope (Karl Zeiss).

RESULTS

The background of the SO and the perikarya of neurosecretory neurons were diffusely and lightly stained red in all sections including the negative controls, while the positive reaction showed a blue or a deep purple granular staining. The diffuse staining increased with the passage of time after the reaction. The increased staining at the edges of a section was not biologically significant. Capillaries were negative for the test (Fig. 1).

Group A. Although some Bergmann glia cells in the cerebellum were mildly stained in some cases, astrocytes extending stained cell processes in all directions were not observed in the hypothalamic area and cerebellum.

A considerable number of cells were clearly stained for GABA-T in the perinuclear zone just dorsal to the SO. A significant number of medium-sized cells were stained in the dorsal part, while more numerous smaller cells of fusiform and multipolar shapes were stained in the ventral part of the perinuclear zone. A few of these smaller cells were located at the dorsal limit of the SO. In rare cases, they appeared to send positive beaded nerve fiber-like structures to the SO. However, in general, the projections of these positive cells to the SO were not detected in the perinuclear zone.

Within the SO, a few small fusiform and multipolar cells showed mild activity in their perikarya and proximal parts of cell processes, although their distal parts and destinations were not detected.

Granular reaction products for GABA-T surrounded unreactive cell bodies of some neurosecretory neurons in discrete groups scattered within the SO.

Group B (Figs. 1-7). Bergmann glia cells were not stained at all in this group. Much fewer cells were stained in group B than in group A. Although a significant number (several cells per section) of medium-sized cells were detected in the dorsal part of the perinuclear zone (Figs. 1-3), most smaller cells in the ventral part of this zone that were clearly stained in group A, were not detected in this group (Figs. 2, 4). In the perinuclear zone, negative images of cells similar to these

stained cells in size and location were observed (Figs. 3, 4). The projections of these GABA-T-intensive neurons to the SO were not detected (Figs. 1-4).

Granular reaction products for GABA-T, sometimes showing a thin thread-like appearance, surrounded the unstained perikarya of some neurosecretory neurons located on the dorsal margin of the SO (Fig. 5) as well as discrete groups widely distributed within the SO (Fig. 6).

GABA-T-intensive intrinsic neurons were not detected within the SO. However, very few small cells of fusiform and multipolar shapes showed mild activity in their perikarya inside the SO in a few cases of group B (Fig. 6).

In the cerebellum, similar results were obtained as those previously described (15-17). The background of the molecular layer was mildly stained whereas Bergmann glia cells were not stained. Small cortical cells and basket cells were mildly stained, while Purkinje cells were moderately stained. The basket formations below the bases of Purkinje cells were intensely stained. Golgi cells showed mild to moderate activity while synaptic glomeruli exhibited mild activity in the granule cell layer. It is to be noted that in deep cerebellar nuclei (Fig. 7), unreactive neuronal perikarya were surrounded by darkly stained thin threads or the granular reaction products for GABA-T (cf. 15).

DISCUSSION

The specificity of the method applied to group B for selectively detecting GABA-T-positive neuronal perikarya should be discussed. Much fewer cells were stained in group B than in group A, where some Bergmann glia cells were mildly stained (17). Astrocytes in the optic tract and cerebellum, and Bergmann glia cells were not stained in group B. The distinct difference observed between the reactions of morphologically similar neurons in the perinuclear zone indicates the specificity of the technique applied to this group. Furthermore, the pattern of staining in the cerebellum was consistent with that previously reported with the same method (15, 17). There is convincing evidence for the correspondence of GABA-T-intensive and GABA neurons in the rat brain (15, 17). The results obtained from group B reliably represent the distribution of GABA-T-intensive,

FIGS. 1-4. Group B (40 mg/kg gabaculine, survival period 15 hr) showing the GABA-T-intensive neurons around the SO.

FIG. 1. Medium-power photomicrograph shows several strongly positive medium-sized neurons (*) in the dorsal part of the perinuclear zone (PZ) just dorsal to the SO (SO), where negative images of many capillaries (c) are seen. $\times 375$

FIG. 2. Higher magnification micrograph than Fig. 1 shows the reaction in the perinuclear zone (PZ). One darkly stained medium-sized neuron (*, A) is located in the dorsal part while the other smaller stained neuron (*, B) (on the different focus level from A) is located in the ventral part of the zone (PZ) close to the SO (SO). Negative images of smaller cells than A (arrows) are seen mostly in the ventral part of the zone (PZ). There are darkly stained thin threads (arrowheads) surrounding the cell body of a neurosecretory neuron at the dorsal limit of the SO (SO) (lower right corner). $\times 600$

FIGS. 3 and 4. The strongly positive medium-sized (*, A) and smaller (*, B) neurons in Fig. 2 are magnified in Fig. 3 and Fig. 4, respectively. Similar neurons (arrows) to them in shape and location are not stained for the reaction. $\times 1,500$

presumably GABAergic neurons in the rat SO. Those obtained from group A are available for complementary results to substantiate the principal findings in group B, because astrocytes were not detected in and around the SO in group A as well.

The 50% decrease in GAD after local injection of kainic acid in the SO suggests that much of this enzyme is in the intrinsic or the adjacent neuronal populations (14). GABA-T-intensive and/or positive neurons in the perinuclear zone found in this study seem to correspond with these anticipated GABAergic neurons. The present findings are possibly supported by the map by Nagai *et al.* (16), who indicated the presence of a few GABA-T-intensive neurons in the SO area with no particular interpretation.

Although no projections of GABAergic medium-sized neurons in the perinuclear zone to the SO were detected, there is some evidence indicating their presence (5, 27, 28). An electrophysiological study has suggested a model of the neuronal circuit of the osmoreceptor-supraoptic nuclear complex in the primate (27). A quantitative electron microscopic study with deafferentation assumed that two thirds of the axon terminals in the SO are of intranuclear origin (28). However, it is quite probable that the deafferentation was made outside the perinuclear zone, so that the data strongly suggest the presence of substantial projections arising from the perinuclear zone to the SO. A histochemical study using horseradish peroxidase (HRP) showed that labeling of some cells in this zone preceded that of all other cells in the rat brain after HRP injection into the SO (5). Accordingly, GABAergic medium-sized neurons in the perinuclear zone probably send their axons to the SO.

Hayward and Vincent (3) have already classified monophasic specific osmosensitive cells into excitatory and inhibitory neurons in the perinuclear zone of awaking monkeys. Some of GABAergic medium-sized neurons, corresponding with these inhibitory neurons, probably inhibit vasopressin cells of the rat. Some small cells showing moderate GABA-T activity in the perinuclear zone are presumably related to the intrinsic neuronal circuit, because a few of them located immediate to the SO appeared to send positive beaded nerve fiber-like structures to it, although they cannot be considered to be GABAergic according to the criteria of Nagai *et al.* (15, 17). However, they are neurons judging from their shapes and the conditions which did not allow the reappearance of GABA-T in astrocytes.

Convincing evidence for the presence of GABAergic intrinsic neurons within the SO was not obtained from the present experiments. However, very few small cells with low GABA-T activity were recognizable in group B. Judging from their

FIG. 5. High power photomicrograph shows the granular reaction products for GABA-T (arrowheads) surrounding unreactive cell bodies of three neurosecretory neurons at the dorsal limit of the SO (SO) at the bottom. $\times 1,500$

FIG. 6. High power photomicrograph shows a mildly positive small multipolar intrinsic neuron at the center (line *). It shows mild activity in its perikaryon and proximal parts of several cell processes, but no activity in its oval nucleus (line N). The cell bodies of nearby neurosecretory neurons are outlined with darkly stained thin threads (arrowheads). $\times 1,500$

FIG. 7. A part of the deep cerebellar nucleus, used here as a positive control, showing the GABA-T reaction under the same conditions as in group B. The perikarya of neurons are lightly stained red, and are surrounded by darkly stained thin threads or the granular reaction products (arrowheads). A part of nerve fibers (line f) shows a positive beaded appearance. $\times 600$

shapes and location, they correspond to a part of intrinsic neurons identified by the Golgi-Cox method in the rhesus monkey, rabbit, and rat (1, 2, 12). They may not be GABAergic neurons, but there is a possibility that they represent a stage of low protein synthesis (4). Further critical studies are needed to settle this problem.

The essentially negative result within the SO is consistent also with some electrophysiological studies (3, 7, 10, 26). Kelly and Dreifuss (7) ruled out the possibility of the presence of inhibitory interneurons in the recurrent collateral system of the rat SO, although their presence was found in the dog and cat (8). Thomson (26) obtained no evidence for the presence of inhibitory interneurons in the rat SO.

Unreactive neuronal perikarya surrounded by granular reaction products for GABA-T were observed in deep cerebellar nuclei and the lateral vestibular nucleus of the rat (15). Similar observations were made in Purkinje cells and neurons of the dentate nucleus and dentate gyrus by immunocytochemistry using GABA-antisera (13, 18, 22). An electron microscopic study using purified GABA-antisera reports that the perikarya are surrounded by many GABAergic axo-somatic synapses in the rat dentate gyrus (13). This observation is in agreement with the concentration of GAD in axon terminals (9, 19, 25). Just as suggested by Nagai *et al.* (15), using the same method as in this study, granular reaction products surrounding the cell bodies of neurosecretory neurons in the present study suggest a localization of GABA-T activity in axon terminals.

It has been generally accepted that oxytocin cells are located rostro-dorsally while vasopressin cells are located ventro-caudally in the rat SO (6, 20, 23). In view of the wide distribution of neurosecretory neurons surrounded by GABA-T-positive axon terminals, it is reasonably assumed that GABAergic medium-sized neurons in the zone inhibit both oxytocin and vasopressin cells.

Léránth *et al.* (11) assumed the presence of intranuclear richly arborizing axons in the rat SO with electron microscopy. Their idea easily accounts for the abundance of GABA-T-positive axon terminals in the SO, which surround the cell bodies of some neurosecretory neurons on the dorsal surface of the SO as well as in discrete groups throughout the SO. These axons probably originate from GABAergic medium-sized neurons in the perinuclear zone just dorsal to the SO.

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