Korean J. Zool. 38:66-73, 1995

Light Microscopic Observations of GABA-Immunoreactive Neuronal Elements in the Dog Basilar Pons

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Putative gamma aminobutyric acid (GABA)-ergic elements in the basilar pontine nuclei were examined in the dog using an antiserum against GABA-glutaraldehyde-protein conjugates and the peroxidase-antiperoxidase method. GABA-immunoreactive neuronal somata in the basilar pons exhibited various morphology with the majority being spindle-shaped or multipolar, while some were spheroidal. The size of GABA-ergic neuronal somata was relatively small (approximately 10-20 μm) in diameter. GABA-immunoreactive neurons were scattered throughout the pontine nuclei, but the midline region of the medial nucleus at the rostral pons, the lateral nucleus at mid-pontine levels, and the ventral nucleus at the caudal pons exhibited a relatively greater concentration of cell bodies. A sparse number of GABA-ergic neurons were observed within the cerebral peduncle and along the ventral borders of the basilar pons adjacent to the middle cerebellar peduncle at the rostro-caudal levels of the pontine nuclei. These observations provide anatomic evidence of how this inhibitory neural element performs its function in the cortico-ponto-cerbellar circuitry.

KEY WORDS: Basilar Pontine Nuclei, GABA, Immunocytochemistry, Intrinsic Neurons

The basilar pontine nuclei (BPN) have a major role in the transfer of information from the cerebral cortex to the cerebellum in order for the animal to perform voluntary motor activities. Recently it was reported that the basilar pons received various descending and ascending informations from a large number of nuclei throughout the central nervous system (for review, see Mihailoff *et al.*, 1989). In addition, the deep cerebellar nuclei (DCN) project collateral fibers to the basilar pons when they send efferent informations into the ventrolateral nucleus of the thalamus, superior colliculus, and inferior olivary nuclear complex (Lee *et al.*, 1989). Thus, it is possible that intrinsic neurons might exist within the pontine nuclei, participating in the integration and the control of the vast majority of inputs before they transfer information into the cerebellum. Morphological evidence suggesting the existence of Golgi type II local circuit-type neurons within the basilar pons has been suggested by Golgi preparations (Mihailoff *et al.*, 1981). Electrophysiological study also reported that the excitation of basilar pontine neurons due to stimulation of the sensorimotor cortex or cerebral peduncle was usually replaced by suppression with some latency and these EPSP-IPSP sequences might indicate the existence of intrinsic neurons within the basilar pons (Sasaki *et al.*, 1970).

The literature contains relatively little report regarding the specific neurotransmitters utilized by basilar pontine neurons and diverse pontine afferent systems. A sparse number of serotonin-containing neurons exist within the basilar pons

(Dupuy and Calas, 1982). Regarding pontine afferent systems, noradrenergic and serotonin-immunoreactive fibers have been identified(Levitt and Moore, 1979; Steinbusch, 1981). Some report suggests that glutamate and/or aspartate might be utilized as neurotransmitters in the corticopontine system (Thangnipon *et al.*, 1983). In contrast with a variety of afferent systems, projection neurons in the basilar pons send all the efferent fibers to the cerebellum and these mossy fiber terminals are cholinergic (Ross *et al.*, 1983). The present immunocytochemical study was, therefore, performed to investigate the distribution of inhibitory GABA-ergic neuronal elements in the basilar pons and to provide additional morphological evidence in support of the presence of local circuit-type neurons in the pontine nuclei.

Materials and Methods

A total of 12 dogs ranging in weight from 1.0-2.0 kg were used in the present study. Nine of the dogs received an injection of colchicine 24-48 hrs prior to sacrifice. Colchicine is known to inhibit axonal transport of neurotransmitters and thus to enhance somatic labeling. About 2-3 μl of 1% colchicine dissolved in 0.1 M sodium phosphate buffer (pH 7.4) was delivered with a 5 μl Hamilton syringe into or just dorsal to the BPN.

Perfusion and fixation

Under deep anesthesia (3.5% chloral hydrate, 10 ml/kg), animals were artificially ventilated using a respirator and perfused through the left ventricle with a brief flush of saline (100-200 ml) followed by 800-900 ml of a cold (4°C) fixative containing 4% paraformaldehyde and 0.5% zinc salicylate in saline. The brain was removed immediately after perfusion and stored in refrigerator overnight.

Immunocytochemistry procedures

The basilar pons and cerebellum were sectioned transversely on a slicetome at 40 μm. Every fifth pontine section and every tenth cerebellar section were collected separately at tissue culture plate containing ice-cold Tris-buffered saline (TBS, 0.05 M, pH 7.6). Sections were treated according to the unlabeled antibody peroxidase-antiperoxidase (PAP) method of Sternberger (1986). Prior to antiserum incubation the sections were treated with a fresh 0.5% solution of hydrogen peroxide in methanol for 10-30 mins to block free aldehyde groups and endogenous peroxidase. Sections were preincubated with 10% normal rabbit serum (NRS) /0.1 M lysine in TBS for 60 mins and then incubated in mouse anti-GABA monoclonal antibody solution (an aliquot of 10 μg/100 μl) at a dilution of 1:100 with 2% NRS at 4°C for 48 hrs. After rinses with TBS, sections were incubated with rabbit anti-mouse IgG (an aliquot of 0.2 mg/100 μl) at a dilution of 1:100 with 2% NRS for 30 mins. After rinses with TBS, sections were then incubated with mouse PAP immune complexes (an aliquot of 1 mg/50 μl) at a dilution of 1:200 with 2% NRS for 30-60 mins, washed, and subsequently reacted with 0.05% diaminobenzidine (DAB)/0.01% hydrogen peroxide in TBS for 5-10 mins. After several rinses with TBS, sections were mounted on slide glasses, dried, and covered with coverslips. The approximate size of the tissue sections and cell bodies within the sections was determined using a hemocytometer under the light microscope.

Both positive and negative control procedures were included in the present immunocytochemical study. As a positive control, the degree of staining at representative GABA-ergic cerebellar cortical and deep cerebellar nuclear neurons was assessed using cerebellar sections which were processed in parallel with basilar pontine sections. Negative controls included the immunocytochemical processing of pontine and cerebellar sections, in the absence of either primary or secondary antisera, PAP reagent or hydrogen peroxide.

Results

In a positive control experiment, only those elements in the cerebellar cortex which have previously been reported to be GABA-ergic, including Purkinje cells and Golgi cells (Oertel *et al.*, 1982) exhibited strong GABA-immunoreactivity (Fig. 1A and B). Some cells in the deep cerebellar nuclei were also GABA-positive (Fig. 1C

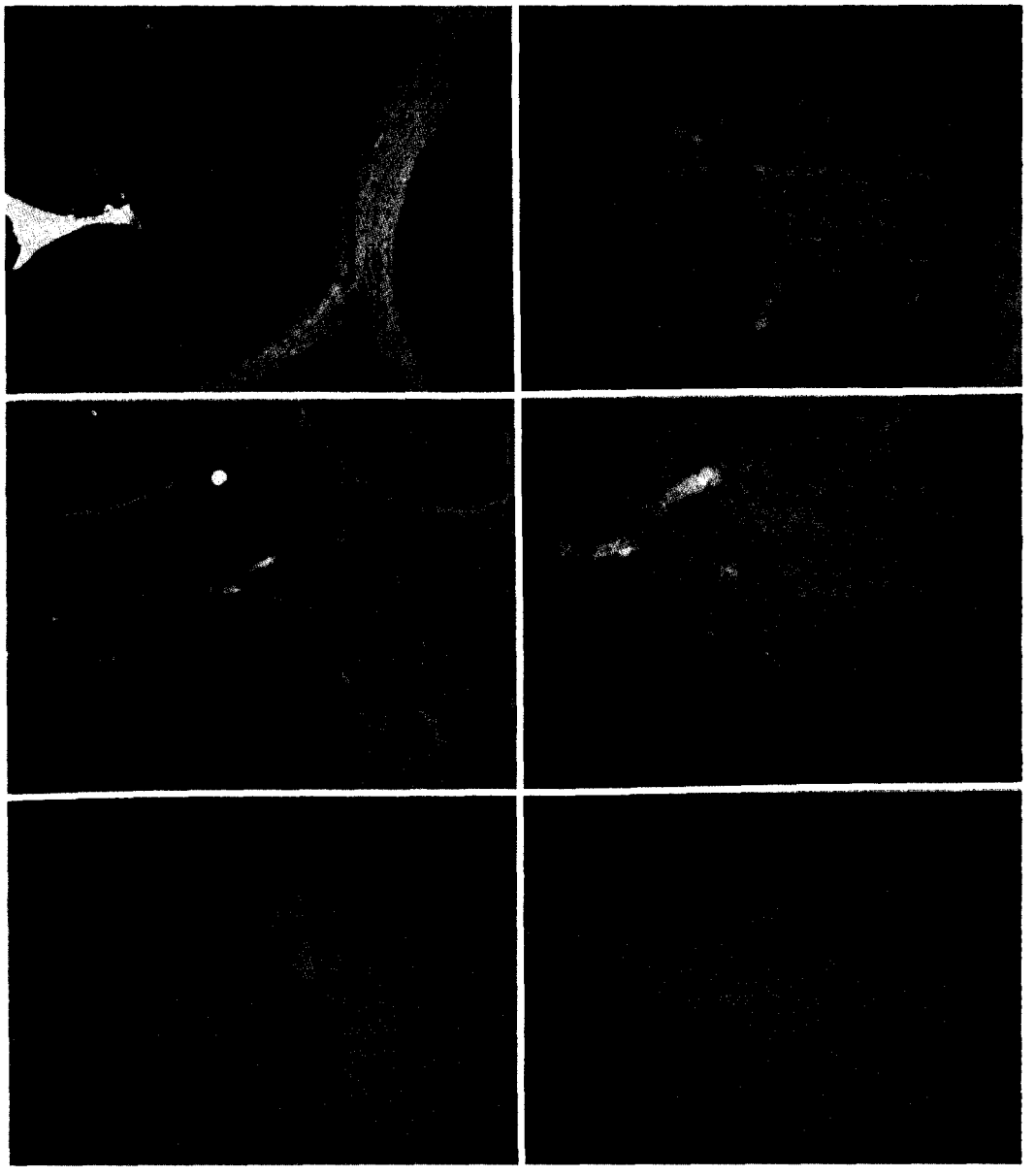

Fig. 1. Low and high magnification views of cerebellar cortical sections reacted with mouse anti-GABA monoclonal antibody are shown in A and B, respectively. Golgi (Go) and Purkinje (P) cells are GABA-positive. Low and high magnification views of GABA-immunoreactive deep cerebellar nuclear cells(arrowheads) are also represented in C and D, respectively. In contrast, cerebellar cortical (E) and pontine (F) sections incubated in the absence of either primary or secondary antisera exhibit no immunoreactivity. Arrows in E specifically indicate unlabeled Purkinje cell monolayer. Bars represent 100 μm. BPN, basilar pontine nuclei; DCN, deep cerebellar nuclei; gran, granular layer; mol, molecular layer; Ped, cerebral peduncle; wm, white matter.

and D), as indicated previously (Nelson *et al.*, 1984). In contrast, all the negative control results were consistent in that neural elements in the cerebellar cortical and basilar pontine sections revealed no immunoreactivity as demonstrated in Fig. 1E and F.

GABA-immunoreactive cell bodies were observed at most pontine sections of the dog. Representative immunolabeled pontine sections and GABA-positive cell bodies within the sections were shown in Fig. 2. GABA-ergic axons and terminals were also distinct at specific areas of the section (Fig. 2B). Additional examples of GABA-immunoreactive cells from the dog basilar pons were shown in Fig. 3. The morphology of the cell body usually varied from spindle-shaped (Fig. 3B, D and E) to multipolar (Fig. 3C), while some cells were more spheroidal (Fig. 3A and F). Although the majority of labeled cells did not exhibit any distinct dendritic morphology, dendritic patterns of multipolar neurons could be followed for some distance (Fig. 3C). In addition to the neuronal somata, GABA-immunoreactive axons and terminals were also observed at some regions of the basilar pons (Fig. 3F). The size of GABA-ergic neurons was relatively small; the diameter of the soma around the cell nucleus was in the range of 10-20 μm in multipolar or spheroidal neurons (Fig. 3A, C and F), whereas the distance between poles along the soma was generally less than 50 μm in spindle-shaped cells (Fig. 3B, D and E).

The distribution and the number of labeled cells

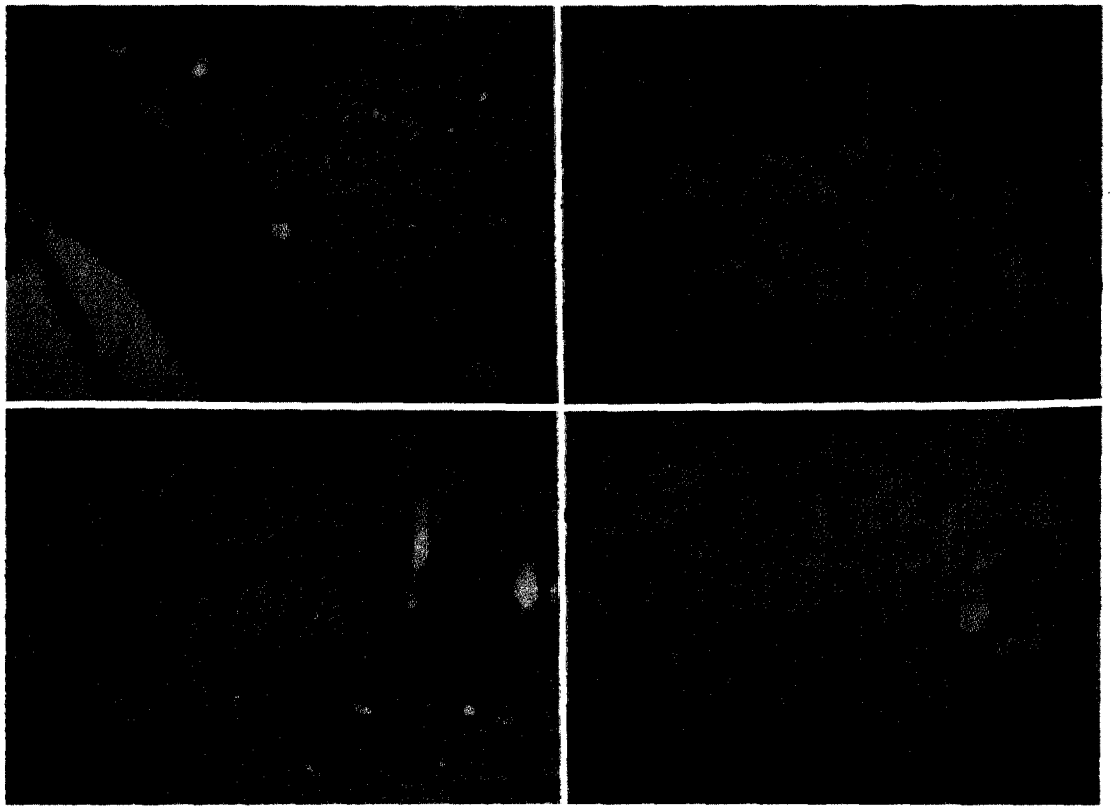

Fig. 2. The presence of GABA-immunoreactive neuronal somata in the ventral borders of basilar pons adjacent to the middle cerebellar peduncle is evident in A (arrows) and the same cluster of labeled neurons is shown at higher magnification in B. GABA-ergic axons and terminals (arrowheads) are also prominent in the section (B). A large number of neurons are GABA-immunoreactive in the ventral nucleus at the caudal pons (C) and two distinct spindle-shaped cells are shown at higher magnification in D (arrows). Bars represent 100 μm. BPN, basilar pontine nuclei; MCP, middle cerebellar peduncle; Ped, cerebral peduncle.

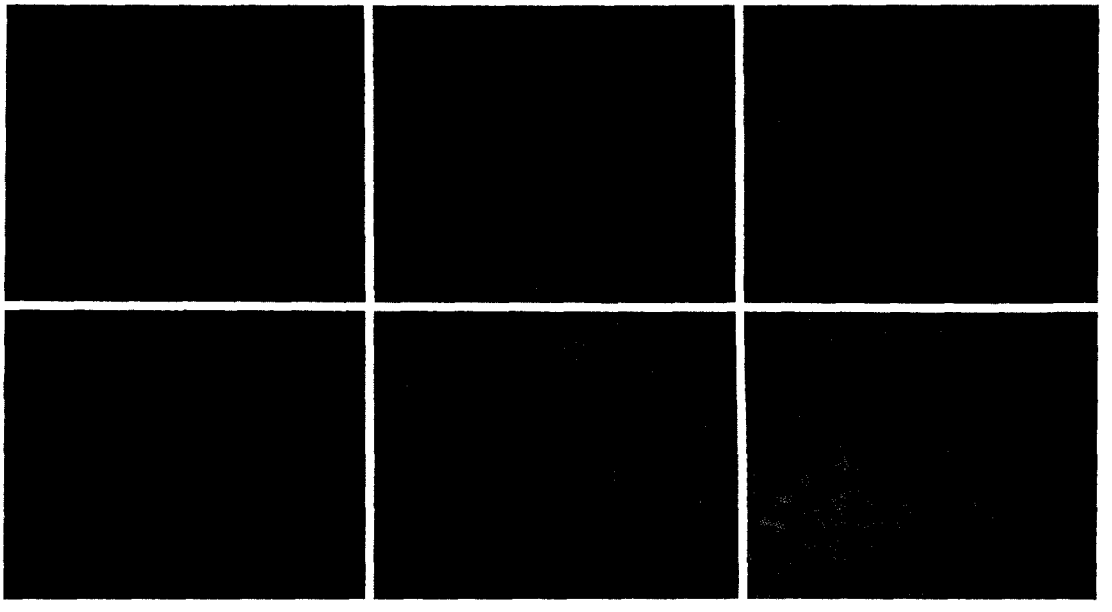

Fig. 3. Examples of GABA-positive neuronal somata found in various regions of the basilar pons (A-F). GABA-immunoreactive axons and terminals (arrowheads) are also evident in the basilar pontine neuropil (F). Bar represents 50 μm.

were relatively consistent in each experiment regardless of whether or not colchicine had been injected. The location of GABA-immunoreactive cell bodies in a representative case (Dog No.8) was shown in Fig. 4. Although labeled neurons were widely distributed throughout the basilar pons, several regions exhibited a relatively greater concentration (Fig. 4, sections 3, 5 and 8: arrowheads). One of the regions was the midline area of the medial nucleus at rostral pontine levels (Fig. 4, sections 2-4). The second pontine area which contained a large number of labeled neurons was the lateral nucleus at mid-pontine levels (Fig. 4, sections 4-7). Lastly, there was a cluster of GABA-ergic neurons in the ventral nucleus at somewhat caudal pontine levels (Fig. 4, sections 7-9). In addition, a few labeled cells were scattered within the cerebral peduncle and the ventral borders of the basilar pons adjacent to the middle cerebellar peduncle (Fig. 4, sections 5-9).

Discussion

The present study has reported for the first time the presence of GABA-immunoreactive neural elements in the basilar pontine nuclei of the dog. Distribution patterns of GABA-ergic neurons were in a general accordance with those reported in the rat (Border and Mihailoff, 1985), whereas the density of labeled cells seemed to be much higher than that in the rat. This seems to reflect an evolutionary increase of local inhibitory neurons in the basilar pons.

As shown in Fig. 2 and Fig. 3, the morphology of the GABA-positive neuronal somata was quite similar to the local circuit-type pontine neurons described in Golgi preparations (Mihailoff *et al.*, 1981). In addition, the diameter of GABA-ergic neuronal soma around the nucleus was relatively small (approximately 10-20 μm) compared with large-sized (25-35 μm) projection-type neurons, as described in the rat (Border and Mihailoff, 1985). This might also be interpreted to suggest that the majority of GABA-positive neurons were Golgi type II local circuit neurons.

GABA-immunoreactive axons and terminals observed in some sections might represent either those of the local circuit neurons whose axon-like processes arborized in the vicinity of neuronal

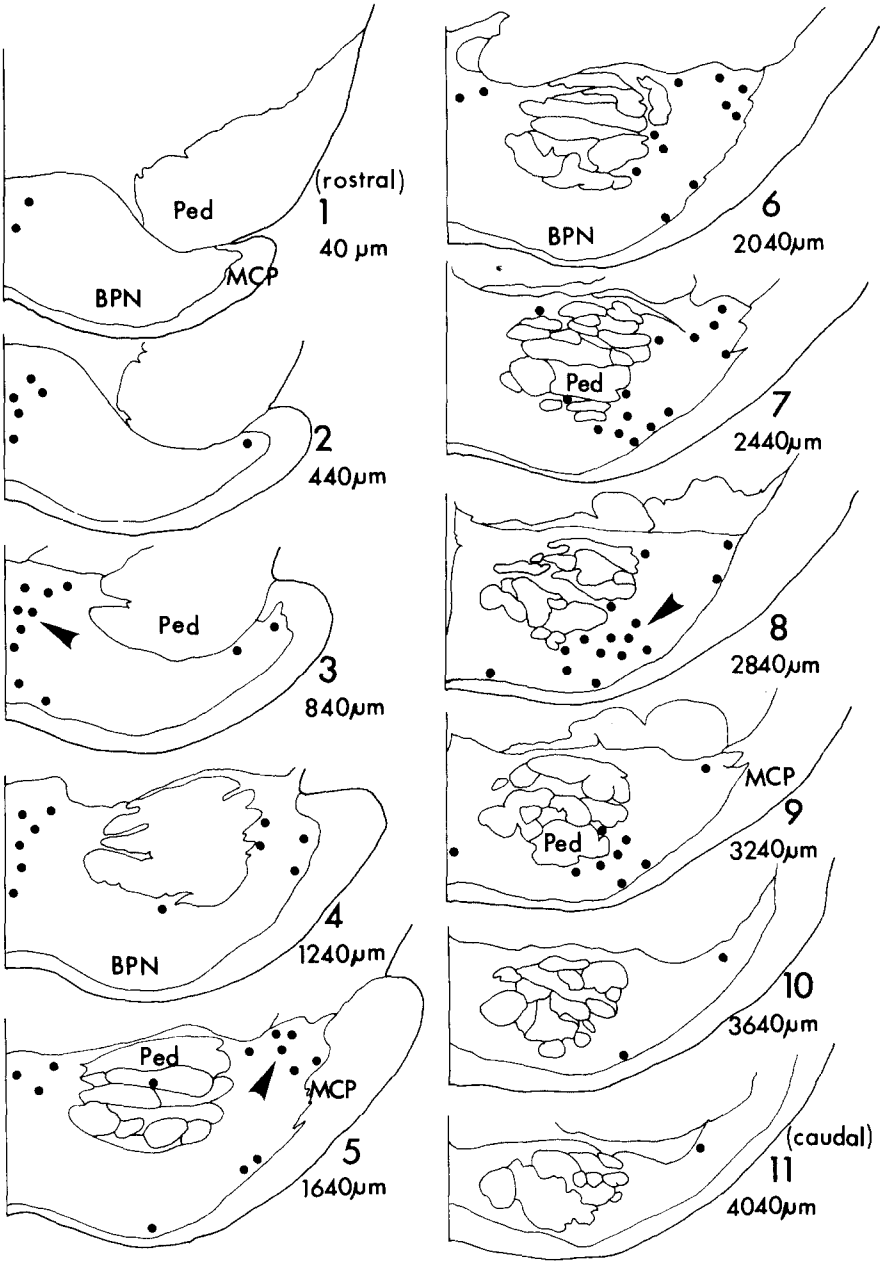

Fig. 4. A rostro-caudal series of unilateral transverse sections (1-11) illustrating the distribution of GABA-immunoreactive neuronal somata within the basilar pons in a representative case (Dog No.8). Filled circles in each section represent approximately 5 to 10 basilar pontine neurons, whereas regions with a group of clustered GABA-positive cells are indicated as arrowheads (sections 3, 5 and 8). BPN, basilar pontine nuclei; MCP, middle cerebellar peduncle; Ped, cerebral peduncle.

somata or axonal fibers of basilar pontine afferent systems (Fig. 2B and Fig. 3F, arrowheads). For the former, the GABA-positive neuronal somata reported in the present study could be the source of axons and terminals. For the latter, brain regions sending those inhibitory axonal fibers might be the zona incerta, the deep cerebellar nuclei, and several brainstem areas, based on the recent double-label study using retrograde transport of wheatgerm agglutinin-conjugated horseradish peroxidase (WGA-HRP) and glutamic acid decarboxylase (GAD)-immunocytochemistry (Border *et al.*, 1986).

Although labeled cells were scattered throughout the basilar pons, there were several regions with increased density of GABA-ergic neural elements (Fig. 4, sections 3, 5, and 8: arrowheads). It should be noted that these regions coincided with areas where various pontine afferent fibers terminate. The first region with increased density of labeled cells was the midline area of the medial nucleus at the rostral pons (Fig. 4, sections 2-4). As previously reported, this region corresponds to where pontine afferents from the mammillary nuclei of the hypothalamus and sensorimotor cortex terminate (Watt and Mihailoff, 1983). Medial and lateral cerebellar nuclei also send a large number of fibers to this region. The second area with a large number of GABA-immunoreactive neurons was the lateral nucleus at mid-pontine levels. This area coincides with where inputs from the superior colliculus, visual and auditory cortices terminate (Burne *et al.*, 1981). A large number of fibers from the lateral cerebellar nucleus also terminate in this area. Finally, a large number of GABA-positive neurons were located in the ventral nucleus at caudal pontine levels, where axonal fibers from the sensorimotor cortex as well as interpositus and lateral cerebellar nuclei terminate (Watt and Mihailoff, 1983). The overlap of terminal areas of pontine afferent systems with the locations of GABA-labeled pontine cells indicates that these pontine neurons might be involved in the integration of various afferent informations and exert certain influences on pontocerebellar projection neurons.

The present immunocytochemical study implies that the inhibitory synaptic mechanism is a part of the functional circuitry of the dog basilar pons. Previous electrophysiological study suggested that inhibitory postsynaptic potentials were consistently observed in the basilar pons following either orthodromic or antidromic activation of pontine neurons (Sasaki *et al.*, 1970). It has been also reported that axo-axonic synapses exist in the dog basilar pons and postsynaptic components in these synaptic complexes represent neural elements of Golgi type II local circuit neurons (Lee, 1993). Thus it can be concluded that GABA-ergic local circuit neurons observed in the present study collect and integrate various afferent informations and convey them into the cerebellar cortex and the deep cerebellar nuclei in order for the animal to preprogram and perform the volitional movement in a precise manner.

References

Border, B.G., R.J. Kosinski, S.A. Azizi, and G.A. Mihailoff, 1986. Certain basilar pontine afferent systems are GABA-ergic: Combined HRP and immunocytochemical studies in the rat. *Brain Res. Bull.* **17:** 169-179.

Border, B.G. and G.A. Mihailoff, 1985. GAD-immunoreactive neural elements in the basilar pontine nuclei and nucleus reticularis tegmenti pontis of the rat. I. Light microscopic studies. *Exp. Brain Res.* **59:** 600-614.

Burne, R.A., S.A. Azizi, G.A. Mihailoff, and D.J. Woodward, 1981. The tectopontine projection in the rat with comments on visual pathways to the basilar pons. *J. Comp. Neurol.* **202:** 287-307.

Dupuy, J.J. and A. Calas, 1982. Distribution of 5-HT accumulating neurons in the rat brain. A radioautographic study after different modes of ^{3}H-5-HT administration. *J. Hirnforsch* **23:** 39-47.

Lee, H.S., 1993. Anatomic evidence suggestive of axo-axonic synapses in the dog pontine nuclei. *Korean J. of Electron Microscopy* **23(3):** 107-114.

Lee, H.S., R.J. Kosinski, and G.A. Mihailoff, 1989. Collateral branches of cerebellopontine axons reach the thalamus, superior colliculus, or inferior olive: A double-fluorescence and combined fluorescence-horseradish peroxidase study in the rat. *Neuroscience* **28(3):** 725-734.

Levitt, P. and R.Y. Moore, 1979. Origin and organization of brainstem catecholamine innervation in the rat. *J. Comp. Neurol.* **186:** 505-528.

Mihailoff, G.A., R.J. Kosinski, S.A. Azizi, and B.G. Border, 1989. Survey of noncortical afferent projections to the basilar pontine nuclei: A retrograde tracing study in the rat. *J. Comp. Neurol.* **282:** 617-643.

Mihailoff, G.A., C.B. McArdle, and C.E. Adams, 1981. The cytoarchitecture, cytology, and synaptic organization of the basilar pontine nuclei in the rat. *J. Comp. Neurol.* **195:** 181-201.

Nelson, B., N.H. Barmack, and E. Mugnaini, 1984. A GABA-ergic cerebello-olivary projection in the rat. *Neurosci. Abst.* **10:** 539.

Oertel, W.H., E. Mugnaini, D.E. Schmechel, M.C. Tappaz, I.J. Kopin, 1982. The immunocytochemical demonstration of gamma-aminobutyric acid-ergic neurons-methods and application, In: Cytochemical methods in neuroanatomy (Chan-Palay, V. and S.L. Palay, eds.). A.R. Liss, Inc., New York, pp.297-329.

Ross, C.D., J.T. Smith, and D. Godfrey, 1983. Regional distributions of choline acetyltrasferase and acetylcholinesterase activities in layers of rat cerebellar vermis. *J. Histochem. Cytochem.* **31:** 927-937.

Sasaki, K., S. Kawaguchi, T. Shimono, and S. Prelevic, 1970. Electrophysiological studies of the pontine nuclei. *Brain Res.* **20:** 425-438.

Steinbusch, H.W.M., 1981. Distribution of serotonin-immunoreactivity in the CNS of the rat. *Neuroscience* **6:** 557-618.

Sternberger, L.A., 1986. Immunocytochemistry, John Wiley Co., New York, p.354.

Thangnipon, W., T. Taxt, P. Brodal, and J. Storm-Mathisen, 1983. The corticopontine projection: axotomy induced loss of high affinity L-glutamate and D-aspartate uptake but not of gamma-aminobutyrate uptake, glutamate decarboxylase or choline acetyltransferase in the pontine nuclei. *Neuroscience* **8:** 449-457.

Watt, C.B. and G.A. Mihailoff, 1983. The cerebellopontine system in the rat. I. Autoradiographic studies. *J. Comp. Neurol.* **215:** 312-330.

(Accepted December 5, 1994)

개의 교핵내 GABA성 신경세포 성분에 관한 광학현미경적 고찰

이현숙(건국대학교 의과대학 의예과)

개의 중추신경계내의 교핵에서 gamma-aminobutyric acid(GABA)를 함유한 신경구조들을 면역세포화학법을 써서 조사하였다. GABA에 대하여 면역반응을 나타내는 신경세포는 형태면에서 대부분 방추형이거나 다극형이었으며, 몇몇은 구형이었고, 세포의 크기는 비교적 작았다(약 10-20 μm). GABA성 신경세포들은 머리-꼬리 방향의 거의 모든 횡절편에서 관찰되었으며, 머리쪽에서는 내측핵의 정중부위에, 중간에서는 외측핵에, 꼬리쪽에서는 복측핵에 다소 밀집하여 존재하였다. 대뇌각과 중소뇌각에 근접한 교핵의 복측경계선에서도 소수의 신경세포들이 관찰되었다. 이와 같은 교핵내 GABA성 신경세포 성분에 관한 광학현미경적 연구는 대뇌-교핵-소뇌의 정보전달과정에서 억제성 신경세포들의 작용기작에 관한 형태학적 근거를 제시한다.