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Immunocytochemical Localization of Glutamatergic Neurons in the Lateral Reticular Nucleus Projecting to Ansiform (Crus I and II) and Paramedian Cerebellar Lobules of the Rat

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I examined the projection of glutamatergic neurons in the lateral reticular nucleus into ansiform (crus I and II) and paramedian lobules in the rat cerebellum using immunocytochemical methods with antiserum against glutamate combined with WGA-HRP histochemistry. The projections of glutamatergic neurons from the lateral reticular nucleus to crus I were most extensive in number among the three injection cases and the majority of projections originated at the dorsal to dorsomedial region of the ipsilateral magnocellular nucleus. Glutamate-immunoreactive cells projecting to crus II were less extensive in number than those projecting to crus I and were mainly localized at the dorsomedial portion of the ipsilateral magnocellular nucleus. Double-labelled neurons projecting to crus I or crus II were also located at ipsilateral subtrigeminal as well as contralateral magnocellular nuclei. Glutamatergic neurons projecting to paramedian lobules were moderate in number and mainly located at the dorsal area of the ipsilateral magnocellular nucleus. A few double-labelled cells were also found at ipsilateral subtrigeminal or contralateral magnocellular nuclei. The present study suggests that glutamate-immunoreactive neurons at the dorsal to dorsomedial magnocellular division of the lateral reticular nucleus may participate in the excitatory control of target neuronal activities at ipsilateral, posterior hemispheric lobules of the rat cerebellum.

It has been well established that the lateral reticular nucleus (LRN) plays an important role in relaying sensory and motor information to the cerebellum. The LRN is also known as a site of the integration of hypothalamic, cardiovascular, and somatic afferent inputs as well as the mediation of cardiovascular responses observed during the activation of sensorimotor system (Blessing, 1990). In addition, recent observations indicate that the LRN may be involved in antinociception via the dorsal horn neurons of the spinal cord (Tavares and Lima, 1994).

The LRN receives inputs from a variety of sources including the spinal cord, the cerebral cortex, the red nucleus, the fastigial nucleus, and parts of the vestibular nuclei (Shokunbi et al., 1986). Among these afferent systems, inputs from the spinal cord have been proven to be extensive and somatotopically organized (Shokunbi et al., 1985). In contrast, the vast majority of LRN neurons send massive projections to the cerebellar cortex. The LRN also sends fibers to other brain regions including the hypothalamic and thalamic nuclei, the periaqueductal gray, and several brainstem nuclei (Payne, 1987).

A series of immunocytochemical studies indicate that lateral reticular neurons are immunoreactive to the choline acetyl-transferase, somatostatin, angiotensin II, neuropeptide Y, and several endogenous, analgesic neuropeptides including substance P, enkephalin, and dynorphin (Satoh et al., 1983; Mansikka, 1995). In spite of the massive anatomical link between the LRN and the cerebellar cortex, the neurotransmitter relating to the reticulocerebellar pathway remains relatively unknown.

In recent years, the transmitter role of glutamate has been well established (Seeburg, 1993). Glutamate is concentrated in synaptic vesicles and synaptic transmission is blocked by glutamate receptor antagonists. In addition, molecular dissection of multiple receptor proteins and their gene families have been reported (Baird et al., 1996). Along with a rapid synaptic transmission, this amino acid can induce prolonged changes in neuronal excitability, such as long-term potentiation and depression. Glutamatergic neurons at the LRN projecting to the cerebellar vermis were identified in the kitten (Wang et al., 1993). The present study was thus undertaken to determine the distribution

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of glutamatergic LRN neurons projecting to the posterior cerebellar hemispheres including ansiform (crus I and II) and paramedian lobules of the rat.

Materials and Methods

A total of 26 albino rats weighing 300-340 g were used in the present study. At least five experimental animals with well-defined injection area were utilized for each injection case of crus I, crus II, or paramedian lobule in the cerebellar hemisphere.

Tracer injection and perfusion fixation

Rats were anesthetized with 3.5% chloral hydrate (10 ml/kg body weight) and placed in a stereotaxic apparatus. After the scalp over the dorsal surface of the head was incised, an opening was made in the skull covering the specific cerebellar hemispheric lobule using an electric drill. Approximately 1-2% wheatgerm agglutinin-conjugated horseradish peroxidase (WGA-HRP, Vector Lab.) in 0.9% NaCl was injected by means of pressure injection. For each injection case, a total of 0.4-0.5 μg WGA-HRP was deposited at a depth of 1.0 mm from the surface of the cerebellar hemispheres over a 30 min period. One day after the injection, 100 μl of colchicine (Sigma) dissolved in 10 μl saline was injected into the cisterna magna of the animal. On the next day, the animal was perfused with 100-150 ml of warm saline followed by 600 ml of cold (4℃) fixative containing 4% paraformaldehyde in 0.1 M phosphate- buffered saline (PBS, pH 7.4). Following perfusion, the brainstem region including the medulla and the cerebellum was removed from the animal and stored in PBS at 4℃.

WGA-HRP histochemistry

Serial transverse sections of the caudal medulla as well as parasagittal sections of the cerebellar injection site were made at the thickness of 50 μm on a vibratome. Every fifth section through the LRN as well as every tenth section through the cerebellar injection site was collected in a 24-welled tissue-culture plate and reacted according to the combined method of nitroprusside-stabilized 3,3′,5,5′-tetramethylbenzidine (TMB) reaction and cobalt/nickel-intensified 3,3′-diaminobenzidine (DAB) reaction as described in detail elsewhere (Lee, 1996).

Immunocytochemistry

Following the WGA-HRP histochemistry, sections were rinsed with saline and processed for glutamate immunocytochemistry utilizing peroxidase-antiperoxidase (PAP) method of Sternberger (1986), which was precisely described elsewhere (Lee, 1997). Sections were washed with 0.05 M Tris-buffered saline (TBS, pH 7.6) and incubated for 5 min in 3% H_2O_2/10% methanol to block endogenous peroxidase activity. Sections were transferred to 10% normal goat serum and eventually incubated in a 1:1000 dilution of the primary antibody solution containing 2% normal goat serum. Lyophilized aliquots of the primary antiserum directed against fixative-modified glutamate were kindly provided by Dr. B. Border (University of Texas Southwestern Medical Center at Dallas). Tissue was incubated for a period of 16-24 h at room temperature followed by incubation in the secondary antiserum solution (1:100, 45 min) containing 2% normal goat serum and goat anti-rabbit IgG. After several rinses with TBS, sections were finally incubated in the rabbit PAP solution (1:100, 60 min) containing 2% normal goat serum. The secondary antiserum and the PAP solution were purchased from Chemicon Inc.. Sections were rinsed with TBS and incubated in cold (4℃) TBS solution containing 0.05% DAB/0.01% H_2O_2 for 6-10 mins. Sections were then thoroughly washed with TBS and observed under a light microscope to identify the reaction product. Positive tissue controls were performed using sections from several brainstem regions including the inferior olivary nucleus which was previously described to have glutamate-immunoreactive neurons (Ottersen and Storm-Mathisen, 1984; Marmo, 1988). For negative controls, the first possibility was to adsorb the primary antibody to the antigen against which it was directed, and then use the adsorbed antiserum in place of the non-adsorbed primary antiserum to abolish the staining. This procedure could not be performed in the present study because of limited availability of purified antigen. The second line of control was therefore executed either by omitting the primary antibody and substituting it with an equivalent dilution of normal serum, or by reacting a series of sections with increasing dilutions of the primary antibody until all staining became lost. Similarly, omission of the secondary antibody or the PAP solution was performed to indicate whether either the reagents or the procedure give rise to non-specific staining.

Results

In positive control experiments, neurons in the inferior olivary nucleus (ION) exhibited glutamate-like immunoreactivity (Fig. 1A and B) as previously reported (Ottersen and Storm-Mathisen, 1984; Marmo, 1988). They were mainly located at the ION contralateral to the injection site (Fig. 1A). Double-labelled neuronal somata at this region were mostly spherical in shape and approximately 8-10 μm in diameter (Fig. 1B). In contrast, negative control experiments which omitted any one of previously mentioned reaction procedures (i.e. primary antibody, secondary antibody, or PAP solution) could not exhibit any immunoreactivity in brainstem regions involving the ION or the LRN.

Representative examples of double-labelled neurons in the LRN were depicted in Figs. 2 and 3. The

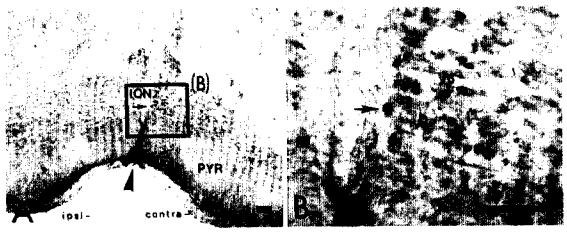

Fig. 1. The projection of glutamate-immunoreactive neuron (arrow) from the inferior olivary nucleus (ION) to the cerebellar hemisphere is shown at low (A) and high (B) magnifications. Arrowhead, midline; ipsi-; contra-; PYR, pyramid. Scale bars=100 μm.

majority of neurons were located at the magnocellular region of the ipsilateral LRN (Fig. 2A and B). The morphology of these cells was pyramidal or spherical and the diameter of somata was in the range of 15-20 μm (Figs. 2 and 3). The WGA-HRP granules within double-labelled cells were black-colored and appeared as either densely aggregated (Fig. 3A) or punctate (Fig. 3B and C). On the other hand, immunoreactive products for glutamate within these neurons looked brown and were homogeneously distributed within the cytoplasm (Fig. 3A and B), although it seemed evident that they were localized outside of the nucleus when the sectioning was performed right at the level of the nucleus (Fig. 3C). In addition to double-labelled neurons, glutamatergic neurons without HRP staining have been observed (Fig. 2B, open arrow). A large number of cells with densely-packed HRP granules have also been identified (Fig. 2B, arrowhead).

The location of double-labelled neurons in the LRN at each injection case including crus I, crus II, or paramedian lobule is represented in Fig. 4, Fig. 5, or Fig. 6, respectively. The glutamatergic projections to crus I were the most extensive in number among the three injection cases and originated mainly from the dorsal to dorsomedial region of the ipsilateral magnocellular nucleus (Fig. 4). Glutamatergic neurons were also located at the medial portion of the ipsilateral subtrigeminal nucleus (Fig. 4, sections 2 and 3).

A few double-labelled cells were identified at magnocellular or subtrigeminal divisions in the contralateral

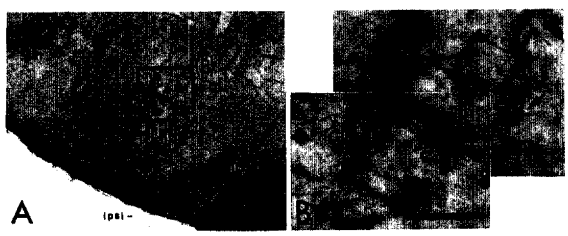

Fig. 2. The projection of glutamatergic neuron(arrows) from the lateral reticular nucleus LRN to crus I is shown at low (A) and high (B) magnifications in a representative case. A Glutamatergic neuron without HRP-labelling (open arrow) as well as a cell with densely-packed, HRP granules (arrowhead) was also depicted. Ipsi-; mc, magnocellular division of the LRN; pc, parvocellular division; PYR, pyramid; st, subtrigeminal division. Scale bars=100 μm.

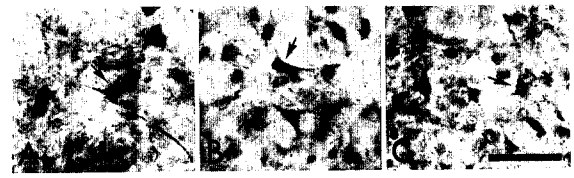

Fig. 3. Representative examples of double-labelled neurons (arrows) at each injection case of crus I (A), crus II (B), or paramedian lobule (C). The majority of these neurons were approximately 15-20 μm in diameter. Scale bar=100 μm.

LRN (Fig. 4, sections 2-6). The projections of glutamatergic neurons to crus II were less extensive in number than the projections to crus I (Fig. 5). Double-labelled neurons were mainly located at the dorsomedial portion of the ipsilateral magnocellular division (Fig. 5). A few double-labelled cells were found at subtrigeminal or parvocellular division in the ipsilateral LRN (Fig. 5, sections 2-4). Additional double-labelled cells

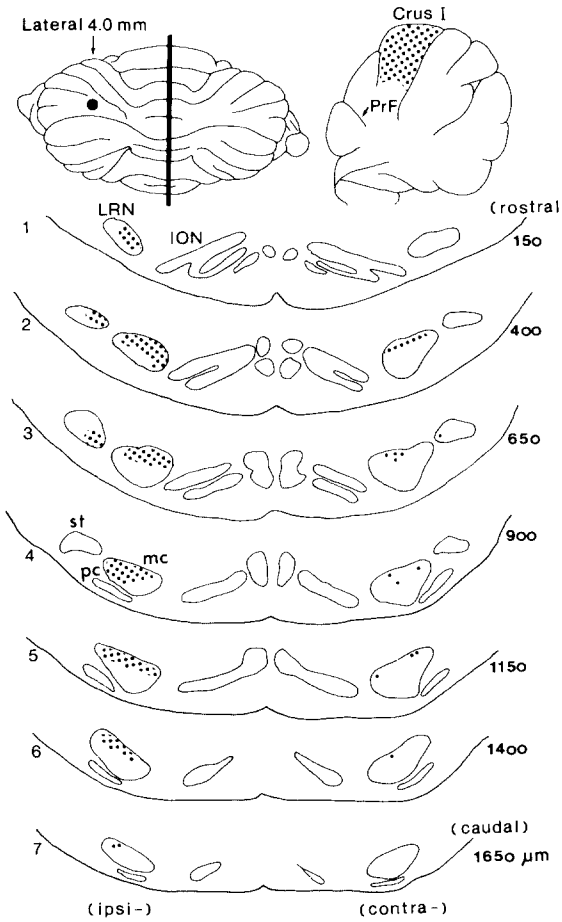

Fig. 4. The injection site of WGA-HRP within crus I (left) and its labelling pattern at parasagittal cerebellar section (right) were shown on the top. The bottom represents a rostro-caudal series of transverse sections (1-7) depicting the location of double-labelled neuronal somata (filled circles) within the LRN in a representative case; mc, magnocellular division; pc, parvocellular division; PrF, primary fissure dividing anterior and posterior lobes of the cerebellar hemisphere; st, subtrigeminal division.

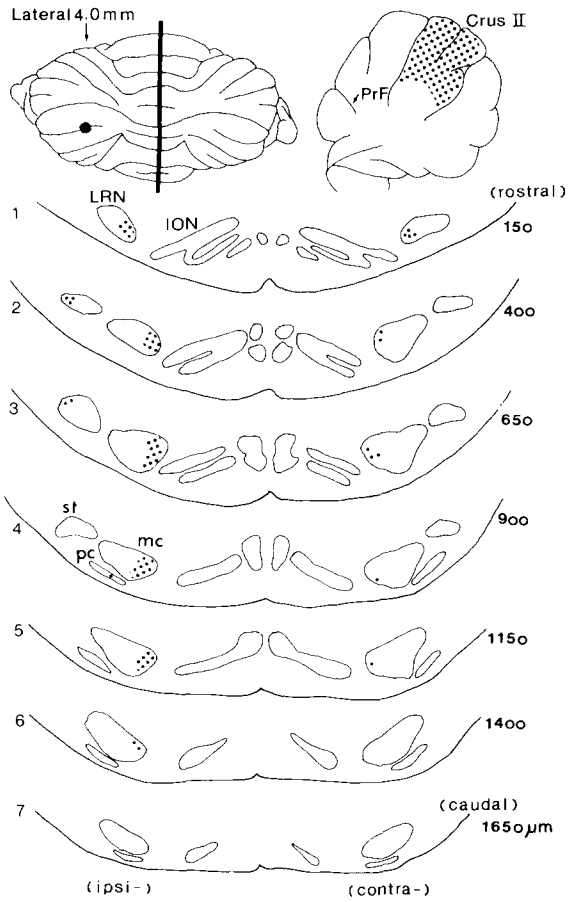

Fig. 5. The injection site of WGA-HRP within crus II (top left) and its parasagittal section (top right) and a series of LRN sections (1-7) with double-labelled neurons (bottom) were diagrammed in a representative case; mc, magnocellular division; pc, parvocellular division; PrF, primary fissure dividing anterior and posterior lobes of the cerebellar hemisphere; st, subtrigeminal division.

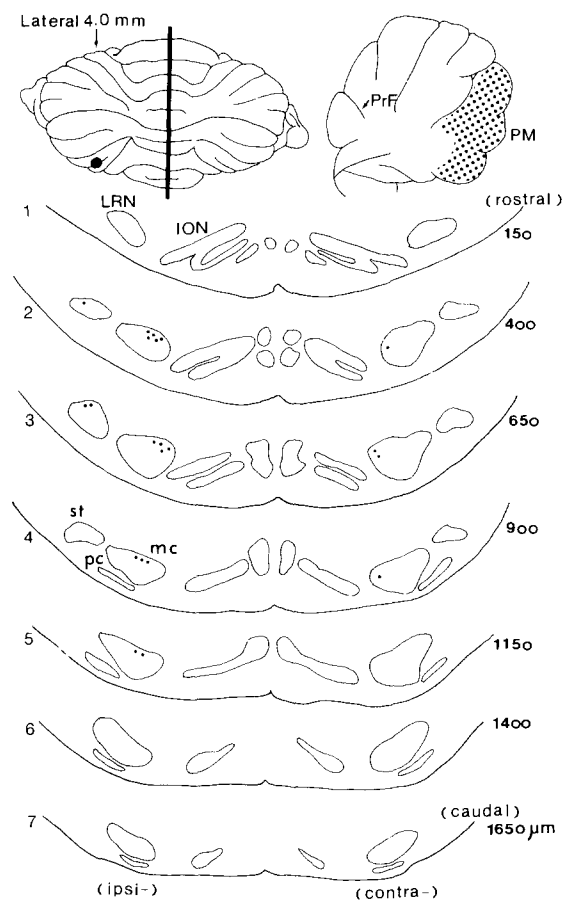

Fig. 6. Drawings representing the injection site of WGA-HRP within the paramedian lobule (PM, top left and right) and plots of selected sections (bottom) showing the distribution of double-labelled cells at the LRN in a representative case; mc, magnocellular division; pc, parvocellular division; PrF, primary fissure dividing anterior and posterior lobes of the cerebellar hemisphere; st, subtrigeminal division.

were also localized at the medial aspect of the magnocellular division in the contralateral LRN (Fig. 5, sections 1-5). The projections of glutamatergic neurons to paramedian lobules were moderate in number and double-labelled neruons were mainly located at the dorsal area of the ipsilateral magnocellular division (Fig. 6, sections 2-5). A few double-labelled neurons were also found at ipsilateral subtrigeminal (Fig. 6, sections 2 and 3) or contralateral magnocellular nuclei (Fig. 6, sections 2-4).

Discussion

The evidence supporting the existence of glutamate-containing, lateral reticulo-cerebellar projection neurons was originally implicated in literature which described the projection from the LRN to the cerebellar vermis in the kitten (Wang et al., 1993). The present study indicated that there existed glutamatergic projections from the LRN to the cerebellar hemispheres including ansiform (crus I and II) and paramedian lobules in the rat.

In addition to double-labelled cells, two other categories of LRN neurons could also be identified in the present study; one population of LRN neurons included glutamatergic neurons without HRP-staining, which might indicate cells projecting to cerebellar cortical areas other than the specific hemispheric lobule employed in the present injection case (Fig. 2B, open arrow). It could also represent a population of neurons that provide excitatory inputs into other projection areas including the hypothalamic and thalamic nuclei, the periaqueductal gray, and several brainstem nuclei (Payne, 1987). The other population included neurons containing densely-packed HRP granules (Fig. 2B, arrowhead). Since black reaction-product might mask the brownish glutamate staining, these cells could represent either double-labelled neurons or reticulo-cerebellar projection neurons utilizing other neurotransmitters. Concerning the latter possi-

bility, previous immunohistochemical studies have indicated that the LRN contains neurons immunoreactive to choline acetyltransferase, somatostatin, angiotensin II, neuropeptide Y, thyrotropin-releasing hormone, calcitonin gene-related peptide and enkephalin (Wang et al., 1993). Further study is therefore needed to determine the distribution of reticulocerebellar projection neurons using other neurotransmitters (Satoh et al., 1983).

The present study revealed that glutamatergic reticular neurons projecting to crus I were the most extensive in number among the three injection cases, whereas projections to crus II and paramedian lobules were moderate (Figs. 4-6). Electrophysiological as well as anatomical evidence suggests that there is little LRN input to the cerebellar cortex outside the classical spinal receiving areas of the anterior lobe and the paramedian lobule in the cat (Clendenin et al., 1974). However, it has been consistently observed that the projection of glutamatergic neurons from the LRN to crus I might be more extensive in number than that to paramedian lobules in the rat (Figs. 4 and 6). Payne (1987) also suggested that there seemed to be a greater LRN input to the anterior part of the posterior lobe hemisphere than to the posterior part.

In general, the projection of glutamatergic neurons from the LRN to cerebellar hemispheres was predominantly ipsilateral in the rat (Figs. 4-6), although a previous report indicated that the projection to cerebellar vermis was rather bilateral in the kitten (Wang et al., 1993). The location of double-labelled neurons in each injection case including crus I, crus II, or paramedian lobule was overlapped at dorsal to dorsomedial region of the ipsilateral magnocellular division at rostral two- thirds of the LRN (Figs. 4-6). These observations were in general accordance with the previous study which had described the lateral reticulo-cerebellar projection pattern in the rat (Hrycyshyn et al., 1982). Species differences, however, were also observed. It has been previously reported that crus II is the only cerebellar region devoid of fibers from the subtrigeminal portion of LRN in the cat (Dietrichs and Walberg, 1979). However, in the present study, a moderate number of glutamatergic neurons at the subtrigeminal nucleus have projected to ipsilateral crus II lobule in the rat (Fig. 5, sections 2 and 3).

Based on the present results, it is speculated that glutamatergic neurons located at the dorsal to dorsomedial magnocellular division of the lateral reticular nucleus may participate in the excitatory control of target neuronal activities at ipsilateral, posterior hemispheric lobules of the rat cerebellum. Confirmation of this notion will require ultrastructural observations demonstrating the existence of glutamate-containing boutons within identified afferent axonal fibers from the LRN to the cerebellar hemispheric lobules. Furthermore, an electrophysiological study measuring the sensitivity of LRN-evoked cerebellar neuronal activity towards iontophoretically-released glutamate antagonists might need to be performed in order to identify the role of glutamate in the regulation of cerebellar hemispheric neurons by the LRN cells.

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