Colocalization of neurotransmitters in the deep cerebellar nuclei

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Summary
An abundance of glycine and glycine receptor immunoreactivities was found in all three parts of the deep cerebellar nuclei. Glycine immunoreactivity was restricted to small neurons throughout most of the deep cerebellar nuclei except for a few large positive neurons in the ventral part of the fastigial nuclei. In addition, glycine immunoreactivity was found in boutons outlining somata of large glycine negative neurons. Complementary to the glycine positive boutons was an intense glycine receptor immunoreactivity on large deep cerebellar nuclei neurons. Comparisons of immunoreactivities for glycine, GABA and aspartate in consecutive one micron sections revealed that many small neurons colocalized glycine and GABA, while some large neurons in the fastigal region colocalized glycine and aspartate.

Ultrastructural investigations revealed glycine receptors on postsynaptic sites of dendrites and somata. Most boutons, which were presynaptic to glycine receptor sites, were filled with small flattened vesicles; however, a small percentage of boutons had round clear or dense core vesicles. Frequently, each bouton apposed multiple active zones on the dendrite or soma. One of these active zones was positive for glycine receptor and another was negative.

This study supports: (1) glycine as a neurotransmitter in deep cerebellar nuclei, and (2) glycine and GABA colocalization in the same cell and bouton, but releasing to different receptor sites on the target neuron. Furthermore, the coexistence of glycine with GABA in the same deep cerebellar neuron may play an important role in controlling the onset and duration of signal transmission.

Introduction
Growing evidence supports the notion that glycine cooperates with GABA as major inhibitory neurotransmitters that hyperpolarize target neurons by opening chloride channels (Aprison & Werman, 1965; Curtis et al., 1968; Maksay, 1990; Ottersen & Storm-Mathisen, 1990). The combination of the rapid inhibitory onset of glycine (Curtis et al., 1968) and the sustained inhibition generated by GABA as the result of a slower onset and longer-duration (Faber & Korn, 1991), may result in temporal modulation of inhibitory patterns.

GABAergic neurons are distributed throughout the CNS, while glycine neurons are abundant only in the brainstem and spinal cord (Aprison & Werman, 1965; Campistron et al., 1986; Wenthold et al., 1988; Wenthold & Hunter, 1990). Immunohistochemical labelling of glycine and GABA in the cerebellum, spinal cord and brainstem revealed the coexistence of these two amino acids in neuronal somata (Osen et al., 1990; Ottersen et al., 1988; Todd & Sullivan, 1990; see additional references in Ottersen & Storm-Mathisen, 1990) and boutons (Ottersen et al., 1988; Triller et al., 1987).

In the cerebellum, interneurons (stellate, basket, and Golgi cells) and Purkinje cells are GABAergic while only Golgi cells and some small neurons in the deep cerebellar nuclei (DCN) are glycineergic (Ottersen et al., 1988; Nelson & Mugnaini, 1989). A weak distribution of glycine receptors in the cerebellar nuclei has been demonstrated by in situ hybridization (Sato et al., 1991) and immunohistochemistry (Araki et al., 1989). However, a complete investigation of glycine and glycine receptor in the DCN is lacking. This communication reports the distribution of glycine and glycine receptors within specific types of neuronal somata, dendrites and axons in the DCN as well as shows glycineergic relationships with aspartate and GABA containing neurons.

Materials and methods
Twenty female white rats (Sprague-Dawley) weighing 180-200 g were perfused for 10 min with 2% paraformaldehyde and 0.5% or 1% glutaraldehyde in 0.09 M phosphate buffer. After removing the brain, the cerebellum and brainstem were immersed in the same fixative for 1–2 h. Cerebella were
sectioned on a vibratome into series of five 30–50 μm sections alternating with one 300 μm section.

The 30–50 μm sections were treated with 1% sodium borohydride to remove unbound aldehyde, and then washed with PBS. Before primary antibody incubation, the tissues were preincubated for 1 h with 5% normal serum (specific for the antibody) to block nonspecific binding of antigens. The unwashed sections were incubated overnight in 0.5% normal serum containing primary antisera: glycine (Chemicon, 1:1000), glycine receptor (clone R7A, 93 kDa, Boehringer Manheim, 1:40), GABA (Arnel, 1:20 000), glutamic acid dehydrogenase (Chemicon 1:1000), aspartate (Arnel, 1:10 000), or c-AMP (1:10 000, Dr Ariano).

After three washes in PBS, these immunoreactions were visualized using the Vector ABC Elite kit and DAB as the chromogen. Primary antisera were omitted in the control sections. For double immunolabelling, GlyR immunoreactivity was processed first and was followed by the immunoreaction for glutamic acid dehydrogenase (GAD) antisera.

For EM, the immunoreacted tissues were washed several times with 0.1 M phosphate buffer, post-fixed with 1% glutaraldehyde and then 1% OsO4. After dehydration in a graded series of alcohol, the sections were embedded in Durcupan between two glass slides that had been coated with a releasing agent (Electron Microscopy Sci., PA). The polymerized plastic wafer was trans-illuminated for punching out specific areas of the DCN. The sections were mounted on resin stubs with 5 minute epoxy resin. Ultrathin sections of tissue, either unstained or double stained with uranyl acetate and lead citrate, were examined under a JEOL electron microscope.

For postembedding LM, 300 μm sections were dehydrated with alcohol and embedded in Durcupan (Fluka Co.). Sections 1 μm thick were cut and mounted on charged glass slides (Fisher Scientific Co.). These sections were etched overnight in 25% sodium ethanolate. Following a thorough wash in absolute alcohol, the tissue was rehydrated to water. Deplasticized tissues were circumscribed with a PAP pen (Daido Sanyo, Japan) to form a droplet well on each slide. The immunoreactions followed the same procedures used for the 30–50 μm sections described above.

The distribution of GABA-, glycine- and aspartate-labelled neurons from consecutive plastic sections were mapped with the aid of a high resolution imaging computer to show the colocalization between labelled cells on different sections. The computer program provided means to tag cells according to the reaction and to display superimposed data from different sections. The perimeter of blood vessels was traced and used as markers for identifying the corresponding regions of consecutive sections.

Results

Light microscopy of glycine and glycine receptors

Coronal sections through the three DCN (dentate, interpositus, and fastigius) were analyzed following immunoreactions for c-AMP, glycine, and GlyR (Fig. 1A-C). In both small and large sized neurons of all three nuclear groups c-AMP gave uniform cytoplasmic reactions, while glial cells did not have noticeable labelling (Fig. 1A).

Glycine immunoreactivity was found in small neurons, axons and terminals throughout all three parts of the DCN (Fig. 1B, C). However in the fastigial nuclei, ventrally-located large neurons were also positive for glycine (Fig. 1B, arrows). In Fig. 2A, the different sizes of glycinergic neurons are segregated visibly into two groups within the fastigial nuclei: large neurons are located ventrally and small neurons, dorsally. Glycine positive boutons were apposed to somata of large neurons lacking glycine immunoreactivity (Fig. 2B, asterisks). They formed beaded profiles outlining somata and dendrites. A few isolated boutons appeared in the neuropile.

Immunoreactivity for GlyR was found primarily in the large neurons located throughout all three parts of the DCN (Fig. 1C). An especially dense pattern of positive large neurons occurred in the dorsal hump of the dentate nuclei (Fig. 1C), distinguishing this region from other parts of the DCN (Fig. 2C). At higher magnifications, the GlyR immunoreactivity was clearly delineated on dendrites and somata of large neurons (Fig. 2D). GlyR-positive neurons were the same size as the glycine negative cells (compare Fig. 2D & B, see asterisks).

Colocalization of transmitters

Adjacent 1 μm sections, which were reacted with aspartate, glycine, or GABA antibodies, had parts of the same cell in each section. Figure 3 (A–C) shows immunoreactions of adjacent sections in the interpositus region. Large neurons are labelled with asterisks and blood vessels with circles. In addition, fourteen small neurons appearing in adjacent sections are identified numerically for easy comparison. All large neurons had intense aspartate immunoreactivity, while small neurons had a weak reaction (Fig. 3A). In contrast, large neurons were negative for glycine and GABA, while some small neurons appeared intense for glycine, GABA or both (Fig. 3B & C).

Quantitation of cells in five sets of serial sections showed a high percentage (approximate 50–70%) of the small neurons displaying both glycine and GABA. Less than 25% of small neurons showed intense staining for glycine and the remaining 25% small neurons revealed GABA immunoreactivity alone.

A similar comparison between aspartate, glycine and GABA reactions in the fastigial nuclei (Fig. 3, D–F) revealed many large neurons containing aspartate (Fig. 3D). Among them, four cells (labelled 1–4) also had glycine immunoreactivity (Fig. 3E), but none of them reacted for GABA (Fig. 3F).

Ultrastructural immunoreactivities of glycine, glycine receptors and GAD

In the DCN, thin sections revealed an abundance of glycine immunoreactive boutons as well as GlyR-immunoreactive sites on dendrites and somata (Fig.