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Distribution of Inhibitory Amino Acid Neurons in the Cerebellum With Some Observations on the Spinal Cord: An Immunocytochemical Study With Antisera Against Fixed GABA, Glycine, Taurine, and β-Alanine

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We have raised antisera against three inhibitory amino acid transmitter candidates: GABA, glycine and taurine. The immunogens were amino acid-glutaraldehyde-carrier conjugates. All sera were purified by immunosorption with the glutaraldehyde-treated carriers, and with one or more amino acid-glutaraldehyde-bovine serum albumin conjugates made from amino acids different from those used for immunization. All purified antisera yielded specific staining that could be abolished by preincubation with glutaraldehyde complexes of the amino acid against which the serum was raised, but not by complexes of other amino acids. The three antisera produced very different staining patterns in the normal cerebellum. Immunoreactivity for GABA occurred in cell bodies and processes of stellate, basket and Golgi cells, and in the axons of Purkinje cells. However, it was low in Purkinje cell bodies and dendrites. Immunoreactivity for glycine was selectively localized in Golgi cells, and was found to co-exist with that for GABA in a subpopulation of Golgi cells. Immunoreactivity for taurine was found in all Purkinje cells, but only in a very small proportion of the neurons in the molecular layer. These results show that the different inhibitory amino acids have selective distributions in the cerebellum.

It is generally assumed that a majority of the inhibitory synapses in the brain use an amino acid as transmitter. GABA and glycine are the amino acids that are best established as inhibitory transmitter candidates. β-alanine and taurine have also been shown to effect inhibition, but the evidence that they are actually released as transmitters is not as compelling as for GABA and glycine.

We are grateful to Dr. Brian Meldrum for supplying brains of Senegalese baboons, to Dr. J. Sturman for sending tissues from taurine-depleted cats, and to Dr. B. Ehinger for providing the β-alanine antiserum. The expert technical assistance of A.T. Bore, J. Knutsen, B. Riber and K. Stentoft (immunocytochemistry), and G. Lothe and C. Ingebrigtsen (photography) is gratefully acknowledged. Thanks are also accorded to Drs. E. Rinvik and K. Osen for critical reading of the manuscript. Supported by the Norwegian Council for Science and the Humanities and the Norwegian Council on Cardiovascular Disease. Requests for reprints should be sent to Ole P. Ottersen, M.D., Anatomical Institute, University of Oslo, Karl Johansgt. 47, 0162 Oslo 1, Norway.

Due to the recent advances in the field of immunocytochemistry (for review, see Ottersen and Storm-Mathisen, 1987) it is now possible to study the distribution of amino acids at an anatomical resolution exceeding by far the maximum resolution of biochemical methods. Indeed, the adaptation of the postembedding immunogold staining technique (Somogyi, Halasy, Somogyi, Storm-Mathisen, and Ottersen, 1986) should in theory enable us to localize amino acids at the organelle level, e.g., in synaptic vesicles.

The methodological principles have been thoroughly described in previous reports (Ottersen and Storm-Mathisen, 1984; Ottersen, Storm-Mathisen, Madsen, Skumlien, and Strömhaug, 1986; Storm-Mathisen, Leknes, Bore, Vaaland, Edminson, Haug, and Ottersen, 1983; Storm-Mathisen and Ottersen, 1986). Glutaraldehyde plays a dual role in our strategy; this cross-linking agent is used to conjugate the amino acids to macromolecular carriers to form the immunization complexes, and is also essential for fixing the amino acid in the tissue. Glutaraldehyde couples the free amino acids in the tissue to brain macromolecules, forming complexes that are similar to those used for immunization and thus recognizable by the specific antibodies. The requirement of glutaraldehyde for the formation of the appropriate epitopes is underlined by the fact that our antibodies show much higher affinities for amino acid-glutaraldehyde complexes than for the free amino acids (Ottersen et al., 1986). The end products of the interaction of glutaraldehyde with amino acids and proteins are highly complex and heterogeneous, and most of them have not been characterized in detail. One class of end products appears to consist of a chain of repeating pyridinium subunits to which the amino acids are attached side by side by their amino groups (Hardy, Nicholls, and Rydon, 1976). In such complexes, the glutaraldehyde-derived "backbone" is likely to maintain the amino acid residues in an array that favours interaction with the specific antibodies.

Specificity

Two amino acids may be structurally very similar and yet exert very different effects on nerve cells. For instance, while glutamate is a potent neuroexcitant, its decarboxylation product, GABA, mediates inhibition. It is therefore essential that the antibodies should be able to discern even very subtle differences in amino acid structure. To what extent is this requirement met? This issue was addressed by screening the antisera against test conjugates made by reacting an amino acid (or a peptide) with a macromolecular extract from rat brain in the presence of glutaraldehyde (Ottersen and Storm-Mathisen, 1984; Ottersen et al., 1986). This strategy was chosen in order to obtain test antigens which would resemble, as closely as possible, the antigens formed in the tissue during glutaraldehyde fixation. As shown in Figure 1, our purified antisera against GABA, taurine and glycine produce intense staining of

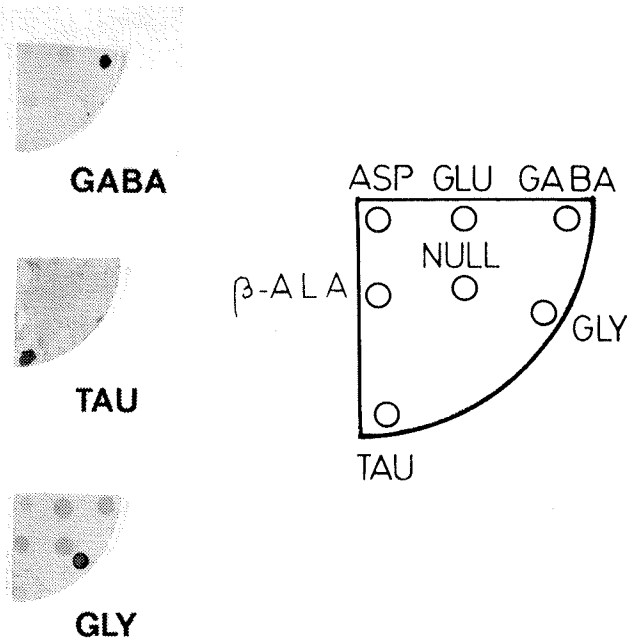


Figure 1: Photomicrographs of test disks incubated with antisera against GABA, taurine, or glycine (left panels). The test disks carried spots containing complexes made by reacting an amino acid (see key in right panel) with brain macromolecules in the presence of glutaraldehyde. The centre spot (null) contained glutaraldehyde-treated protein (no amino acid added). There is selective staining of the complexes containing the amino acid used in the immunogen.

conjugates prepared form the respective amino acids, but show low affinity for other amino acids and for glutaraldehyde treated protein. Selective staining in this model system was also obtained with an antiserum against β-alanine. (The latter serum was raised by B. Ehinger and purified in our laboratory.) More than 40 small molecular compounds have been tested in this way (for a complete list see Ottersen et al., 1986). The test results suggest that our antisera should be useful for demonstrating the specific distributions of individual amino acids in glutaraldehyde-fixed tissue.

Since the first report on amino acid immunocytochemistry appeared (Storm-Mathisen et al., 1983) this technique has been taken up by numerous laboratories, and sera have been produced against GABA (e.g., Hodgson, Penke, Erdei, Chubb, and Somogyi, 1985; Seguela, Geffard, Buijs, and Le Moal, 1984; Wenthold, Zempel, Parakkal, Reeks, and Altschuler, 1986), glycine (e.g., Campistron, Buijs, and Geffard, 1986; Pourcho and Goebel, 1985) and taurine (e.g., Campistron, Geffard, and Buijs, 1986; Yoshida, Karasawa, Ito, Sakai, and Nagatsu, 1986).

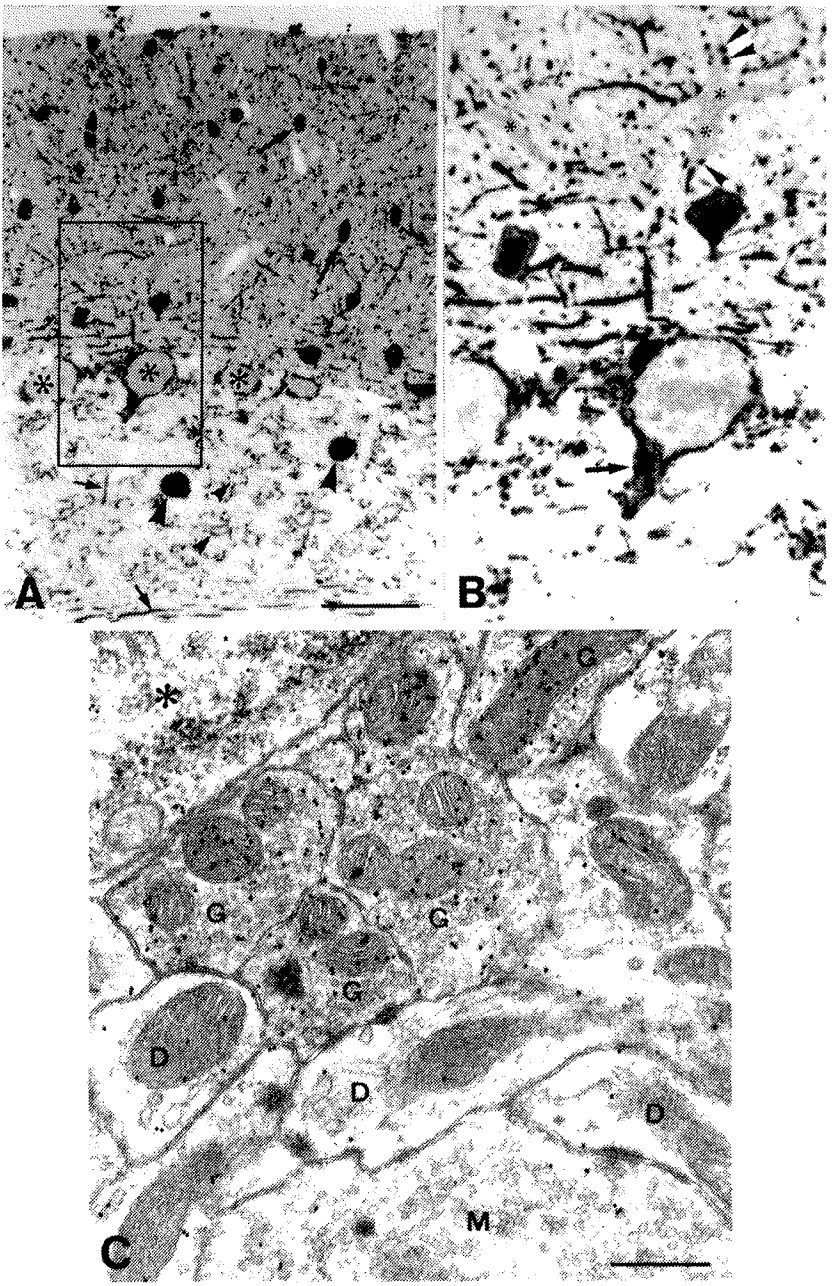
Immunocytochemical Application

In the following we will largely focus on results obtained in the cerebellum. GABA, glycine and taurine have all been implicated as cerebellar transmitters. The relatively simple structure of this part of the brain allows the amino acids to be localized to identified types of cells by means of specific antibodies.

GABA

In the cerebellar cortex, GABA-like immunoreactivity (GABA-LI) occurs in cell bodies and processes of stellate, basket and Golgi cells (see Figure 2; also see Gabbott, Somogyi, Stewart, and Hamori, 1986; Ottersen and Storm-Mathisen, 1984; Seguela, Gamrani, Geffard, Calas, and Le Moal, 1985; Somogyi et al., 1985). The fourth class of inhibitory cerebellar neuron, the Purkinje cells, displayed very low GABA-LI in the somata. However, the Purkinje cell axons took up the staining soon after leaving the cell body, and intense immunostaining was found in the neuropil of the deep cerebellar nuclei where they terminate (not illustrated). Postembedding immunogold labelling at the electron microscopical level revealed that a majority of the axon terminals contacting somata and dendritic stems in the deep cerebellar nuclei were labelled for GABA (Ottersen, Madsen, et al., 1988). The immunopositive terminals typically had flattened vesicles and established synaptic contacts that were symmetric—or of a transitional type. The morphological features of Purkinje cell terminals are not distinctive enough to prove their identity: however, since these terminals are responsible for the majority of the contacts on somata and dendritic shafts (Chan-Palay, 1977), they must have contributed to the population of labelled terminals in our material. It is relevant in this context that in the cerebellar nuclei of mutant mice deficient in Purkinje cells, the number of boutons immunostained for glutamic acid decarboxylase (GAD) is only 15% of normal value (Wassef, Simons, Tappaz, and Sotelo, 1986).

Figure 2: Distribution of GABA-LI in Durcupan-embedded sagittal sections of rat cerebellum (vermis). A: Photomicrograph of postembedding-stained 0.5 μm section showing intense labelling of Golgi cell bodies (large arrowheads) and circularly arranged dots suggestive of Golgi cell terminals (small arrowheads) in the granule cell layer, and of neurons (large arrows) in the molecular layer. The immunoreactivity is low in the Purkinje cell somata (asterisks), but is high in the Purkinje cell axons (small arrows). Frame shows area enlarged in B. B: Note strong staining of the basket cell plexus and pinceau (arrow), and weak staining of the Purkinje cell dendrites (asterisks; unlike other staining not blocked by absorption of serum with GABA-glutaraldehyde complex) which are contacted by immunoreactive dots (arrowheads) that probably represent stellate cell terminals. C: Electron micrograph of ultrathin section processed with the postembedding immunogold labelling technique (Somogyi et al., 1986). The gold particles indicative of GABA-LI are concentrated over Golgi cell terminals (G), but are rarely encountered in cell bodies (asterisk) and dendrites (D) of granule cells, and in mossy fibre terminals (M). GABA antiserum 26, diluted 1:100 (A,B) and GABA antiserum 33, diluted 1:200 (C). Bars: A, 100 μm; C, 0.4 μm. ⟶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Glycine

The glycine antiserum stained only a single neuronal type in the cerebellar cortex of the Wistar rat, i.e., the Golgi cell and its processes (see Figure 3A, 3C).[1] This cell type seems to be the predominant glycine-accumulating neuron in the cerebellum (Ottersen, Davanger, and Storm-Mathisen, 1987; Wilkin et al., 1981).

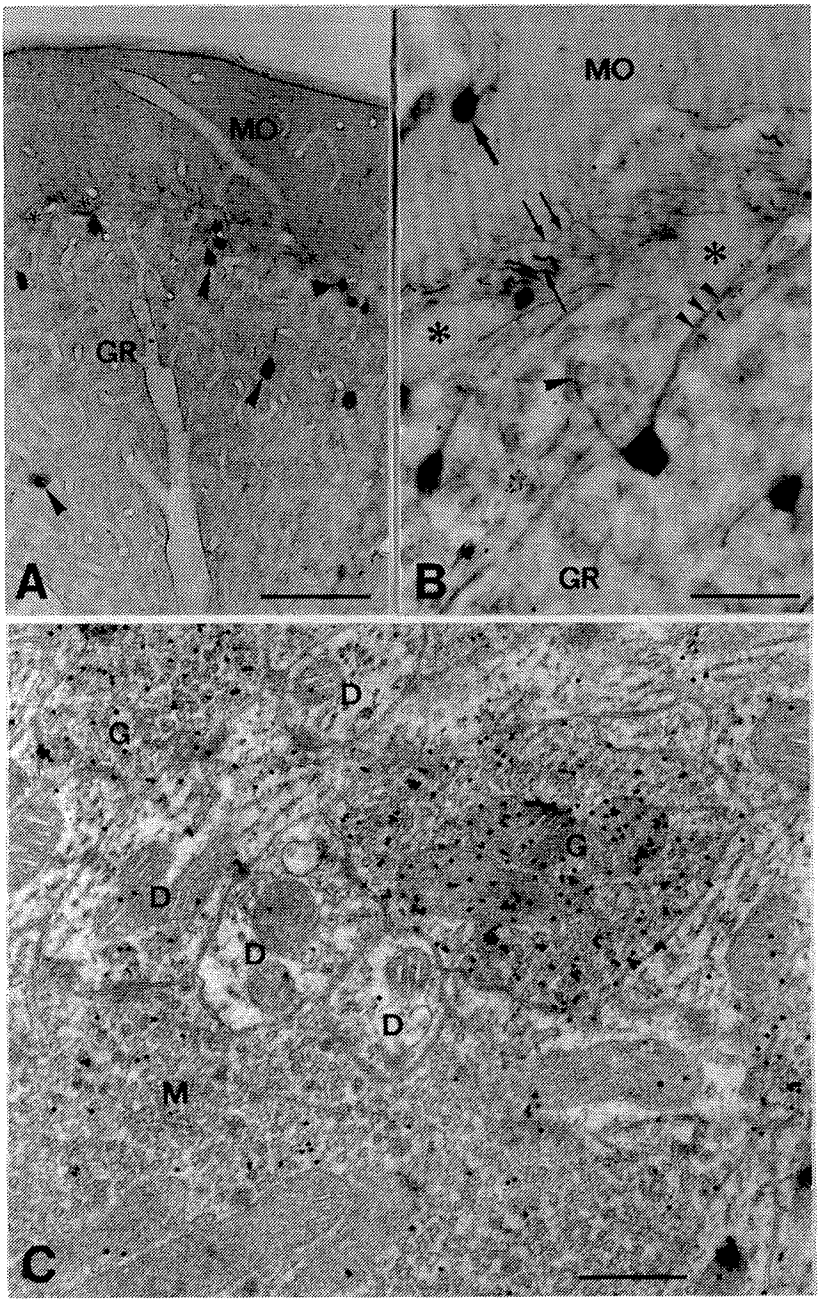
The distribution of glycine-LI in the cerebellum of cat and baboon is largely similar to that in rat. However, in the baboon the glycine antiserum stains a few additional neurons in the molecular layer (Figure 3B). The identity of these neurons is unclear. Although their somata and dendritic trees are reminiscent of basket neurons, we were never able to observe staining of the basket plexus. It is possible, therefore, that the immunostained neurons in the molecular layer represent Golgi cells that have been displaced into the molecular layer during development (the "displaced stellate cells" of Ramón y Cajal, [1911]). Neurons resembling Lugaro cells were also stained (Ottersen, Davanger, et al., 1987).

Colocalization of GABA and Glycine?

Do GABA-LI and glycine-LI occur in overlapping or in separate populations of Golgi cells? After treatment of alternate Vibratome sections with a GABA antiserum or a glycine antiserum, we found that a proportion of the Golgi neurons that had been cleaved by the plane of section exhibited immunoreactivities for both amino acids (Ottersen, Davanger et al., 1987). Similar experiments carried out on adjacent 0.5 μm plastic embedded sections indicated that a majority (about 70%) of the Golgi cells contained GABA-LI as

Figure 3: Distribution of glycine-LI in sagittal sections of rat (A, C) and baboon (B) cerebellum (vermis). A: Vibratome section processed free-floating shows intense labelling of Golgi cells (arrowheads) in the granule cell layer (GR). There is no concentration of immunoreactivity in the Purkinje cells (asterisks), and immunostained neurons in the molecular layer (MO) were extremely rare. B: An immunopositive Golgi cell shows a dendrite (small arrowheads) extending into the molecular layer, and a thinner process (axon?) which appears to engage in a glomerulus-like structure (large arrowhead). In contrast to the rat, the baboon exhibits a few labelled neurons in the molecular layer (large arrow). Note the presence of thick and thin immunoreactive fibres (thin arrows) in the deep part of the molecular layer (origin unknown). Asterisks indicate Purkinje cell bodies. Vibratome section. C: Electron micrograph of ultrathin section processed similarly to that in Figure 2C. As for GABA-LI, glycine-LI is high in the Golgi cell terminals (G), and low in granule cell dendrites (D) and mossy fibre terminals (M). Glycine antiserum 31 diluted 1:250 (A,B) or 1:300 (C). Bars: A, 200 μm; B, 100 μm; C, 0.4 μm.

[1]Campistron, Buijs, and Geffard (1986) found glycine-LI in basket neurons as well as in Golgi neurons in the rat. These authors used slightly different procedures for raising of sera and immunocytochemistry. Also, since no information is given about the rat strain used, the possible influence of strain difference cannot be excluded.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well as glycine-LI (see Figure 4). An analysis of test conjugates incubated together with the sections confirmed the specificity of the immunostaining. Further, with both types of material, the staining with the glycine antiserum was abolished after preincubation with glycine-glutaraldehyde complexes, whereas GABA-glutaraldehyde complexes in similar concentrations had no effect. Conversely, labelling of sections with the GABA antiserum was completely inhibited by GABA-glutaraldehyde complexes but unaffected by complexes of other amino acids including glycine. These control experiments strongly suggest that the presence of glycine-LI and GABA-LI in the same cell bodies is not an artifact due to crossreactivity, but does reflect a coexistence of the two amino acids.

A close examination of the immunostained semithin sections suggested that glycine-LI and GABA-LI were also colocalized in a proportion of the Golgi cell terminals (Figure 4). This was confirmed by electron microscopic analyses of ultrathin sections obtained from the same blocks of tissue as the semithin sections and treated according to the postembedding immunogold staining procedure (Ottersen, Storm-Mathisen et al., 1988). As for light microscopy, inhibition experiments with amino acid-glutaraldehyde complexes ruled out the possibility that our observations should have been confounded by crossreactivity. Further, the specificity of the immunocytochemical reaction was monitored by means of test conjugates incubated together with the tissue specimens (Ottersen, 1987a).

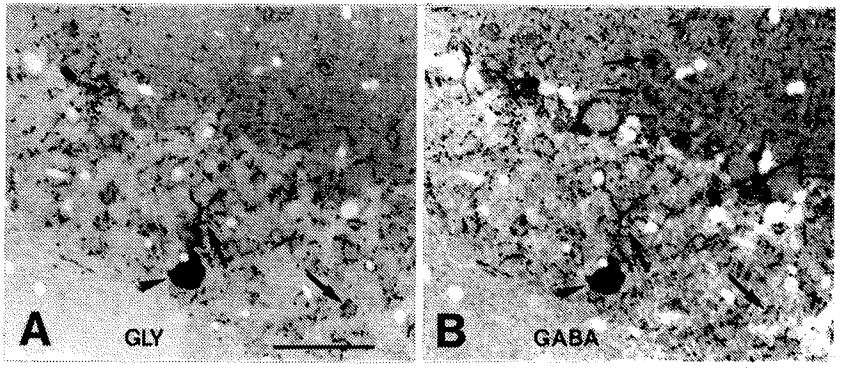

Figure 4: Photomicrographs of postembedding stained 0.5 μm adjacent sagittal sections of rat cerebellar cortex (vermis). The Golgi cell indicated by an arrowhead contains glycine-LI (A) as well as GABA-LI (B). On closer inspection it seems as if the two immunoreactives also coexist in the same glomeruli and even in the same Golgi terminals (thick arrows). This was confirmed by electron microscopy (not shown). GABA-LI, but no glycine-LI, occurs in neurons (thin arrows) and nerve terminals of the molecular layer. Glycine antiserum diluted 1:50 (A), GABA antiserum 26 diluted 1:100 (B). Bar: 100 μm.

Do the Renshaw Cells Contain Glycine-like Immunoreactivity?

To further substantiate the selectivity of the glycine antiserum we applied the serum to sections of spinal cord which contain the most classical of all putative glycinergic neurons: the Renshaw cell. Only two to ten medium-sized neurons were stained in each Vibratome section of cat cervical spinal cord (see Figure 5); these were found ventrally in lamina VII, corresponding to the location of the Renshaw cells as defined by electrophysiological recording followed by intracellular injection of horseradish peroxidase (Lagerbäck and Kellerth, 1985). The size and morphology of the immunoreactive neurons were also compatible with their identity as Renshaw cells. Some small neuronal cell

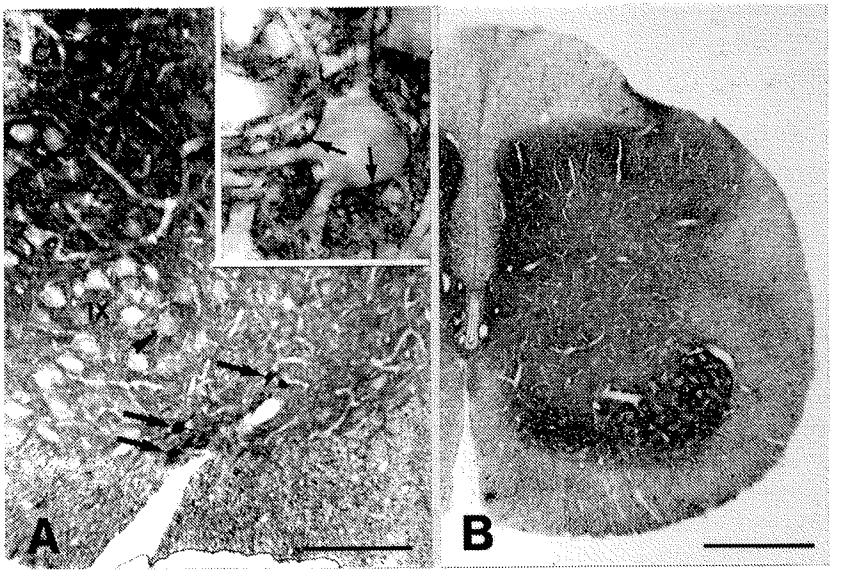

Figure 5: Photomicrographs showing the distribution of glycine-LI in the cervical spinal cord of a four day old kitten (transverse Vibratome sections). A: Immunopositive neurons (arrows) occur ventrally in lamina VII, corresponding to the location of the Renshaw cells. The large motor neurons situated in lamina IX more laterally (i.e., to the left) are unstained but are outlined by immunoreactive dots (small arrows in inset) that probably represent nerve terminals. Arrowhead indicates neuron shown in inset. B: Low power photomicrograph of opposite half of spinal cord at the same level as in A. Note that the immunostaining in the gray matter neuropil is more intense in ventral regions, particularly corresponding to lamina IX, than more dorsally. Scattered small stained perikarya can be seen, particularly in the dorsal horn. The lateral and ventral funiculi show significant labelling due to the presence of numerous immunoreactive nerve fibres. In contrast, the dorsal funiculus is virtually unstained. Glycine antiserum 31, diluted 1:200. Bars: A, 200 μm; B, 500 μm.

bodies were also stained, particularly in the dorsal horn (Figure 5B). Cell bodies showing the size and location typical of motoneurons were invariably unstained, but had numerous immunoreactive bouton-like dots apposed to

their surfaces (Figure 5A). The neuropil staining was somewhat more intense in the ventral than in the dorsal gray matter (Figure 5B), matching biochemical data (Aprison and Werman, 1965; Graham, Shank, Werman, and Aprison, 1967).

Glycine-LI has also been demonstrated in the commissural interneurons in the spinal cord of Xenopus laevis (Dale, Ottersen, Roberts, and Storm-Mathisen, 1986). The latter neurons have been subjected to detailed physiological and pharmacological studies which strongly suggest that glycine is their actual transmitter. Our GABA antiserum 26 did not stain the commissural interneurons in the frog spinal cord; nor did it label neurons in the cat spinal cord that (according to size, shape or position) could represent Renshaw cells.

Taurine

Taurine occurs in high concentrations in brain tissue and may serve multiple metabolic roles in addition to its possible role as a transmitter. In the case of the cerebellum, it has been suggested on physiological and pharmacological grounds that taurine mediates inhibition in the stellate to Purkinje cell synapse (Okamoto, Kimura, and Sakai, 1983a, 1983b). However, immunocytochemistry with antisera against fixed taurine has failed to provide evidence for a concentration of this amino acid in stellate cells (see Figure 6A; also see Madsen et al., 1985; Yoshida, Karasawa, Ito, Sakai, and Nagatsu, 1986). Indeed, quantitative electron microscopic studies with the postembedding immunogold technique (Ottersen, 1987b) have shown that the taurine-like immunoreactrivity of stellate cell terminals (as expressed by the density of gold particles above them) is only about 10% of that of the Purkinje cell somata and dendrites, which display the highest immunoreactivity of all elements in the cerebellar cortex (see Figure 6A, 6B). The Purkinje cell terminals in the deep cerebellar nuclei are even more intensely labelled than the parent cell bodies (see Figure 7): in one particular experiment (Ottersen, 1987b) the density of gold particles indicative of taurine-like immunoreactivity was 60% higher over Purkinje cell terminals than over Purkinje cell somata. The Purkinje cell is the only neuronal cell type in the cerebellum with a considerably higher concentration of taurine-LI in the terminals than in the somata. GABA-LI and taurine-LI have been shown to coexist in the same Purkinje cell terminals (Ottersen, Madsen et al., 1988).

The specific nature of the taurine antiserum was corroborated by control experiments similar to those mentioned above in connection with GABA and glycine immunocytochemistry. To further substantiate our claim that the staining reflects the *in vivo* presence of taurine, we applied our taurine antiserum to cerebellar sections of kittens deprived of taurine both pre- and postnatally. Cats have poor synthetic capacity for taurine, and show, in the

absence of an exogenous supply, a pronounced decrease in the biochemically recorded taurine level in the brain (Sturman, Moretz, French, and Wisniewski, 1985). These brains also show a dramatic decrease in the immunostaining for taurine (not illustrated).

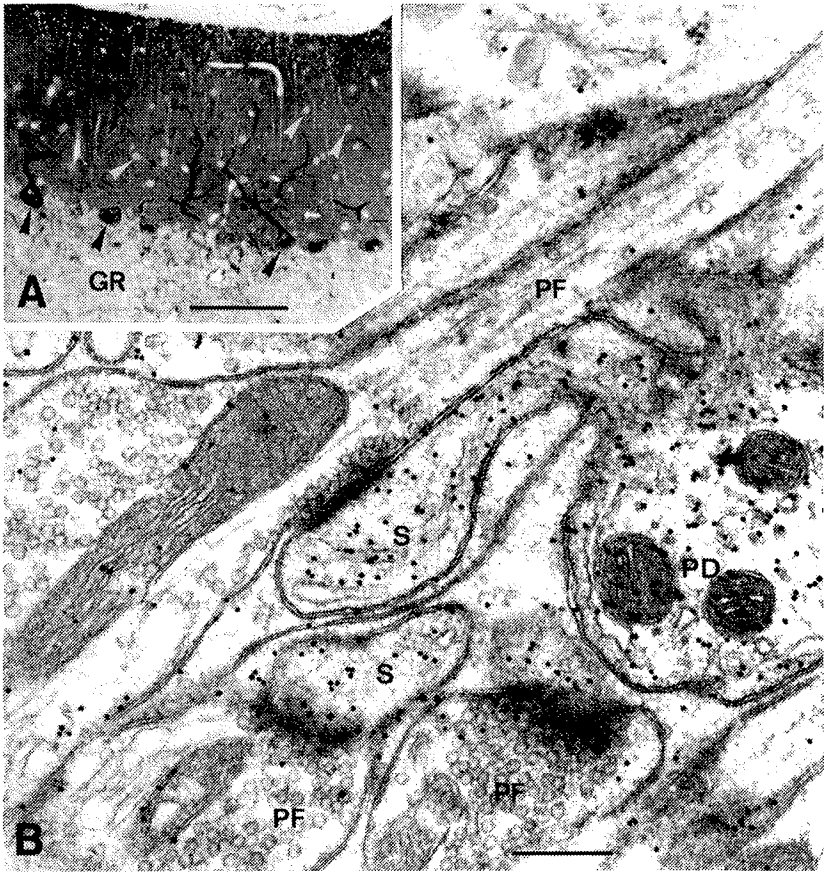

Figure 6: Distribution of taurine-LI in postembedding stained Durcupan-embedded sagittal sections of rat cerebellum (vermis). A: Photomicrographs of 0.5 μm section showing intense labelling of Purkinje somata (black arrowheads) and dendrites. The interneurons (white arrowheads) and the radially oriented Bergmann glia (small arrows) in the molecular layer-(MO) are virtually unstained. The granule cell layer (GR) contains few labelled elements. B: Electron micrograph from ultrathin section obtained from the same block of tissue as in A and treated according to the immunogold labelling procedure (cf., Figure 2C). Numerous gold particles appear over Purkinje cell dendrites (PD) and spines (S) whereas the parallel fibre terminals (PF) are weakly labelled. Taurine antiserum 20 diluted 1:1000 (A) or 1:500 (B). Bars: A, 200 μm; B, 0.3 μm.

β-alanine

A serum against β-alanine (provided by B. Ehinger and purified in our laboratory) mainly labelled glial cells when applied to sections of the cerebellum. Particularly strong staining was observed at the site of the Bergmann cell bodies (Ottersen, Davanger, et al., 1987). Thus, our immunocytochemical data do not support a transmitter role for β-alanine in the cerebellum.

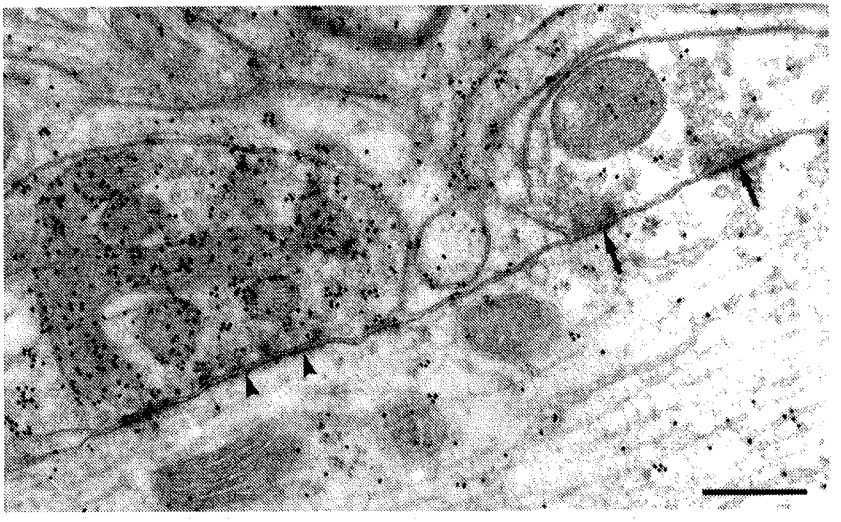

Figure 7: Electron micrograph showing taurine-LI in the dentate nucleus of rat (same section and immunolabelling procedure as in Figure 6B). Note the intense labelling of bouton establishing symmetric contact (arrowheads), and very weak labelling of bouton engaged in asymmetric junction (arrows). The postsynaptic dendritic stem shows modest labelling. The immunopositive bouton, which probably represents a Purkinje cell terminal, also contained GABA-LI, as judged from an adjacent section incubated with an antiserum against GABA (not illustrated). Bar: 0.4 μm.

Discussion

The three most important inhibitory amino acids—GABA, glycine and β-alanine—are structurally very similar, differing only by the number of methylene groups they contain. Yet, with the immunocytochemical technique described here it is possible to distinguish between them, and also to selectively recognize taurine. Each inhibitory amino acid shows a unique pattern of distribution in the cerebellum. However, in a few instances the immunocytochemical data do suggest a colocalization of two inhibitory amino acids in the same neuron. We have provided evidence that GABA occurs together with glycine in a subpopulation of cerebellar Golgi cells, and that at

least some of the Purkinje cell terminals appear to be enriched in GABA as well as taurine. While coexistence of two or more neuroactive substances in the same neuron seems to be a common phenomenon in the CNS, the usual constellation is that of a "classical" transmitter coexisting with a neuroactive peptide (Hökfelt et al., 1980), rather than with another "classical" transmitter.

What could be the functional significance of a colocalization of GABA and glycine? Both amino acids are thought to open chloride channels via specific receptors. Recent evidence suggests that although GABA and glycine may act on a common chloride channel, they have different effects on its conductance states (Barker, Dufy, and McBurney, 1986; Hamill, Bormann, and Sakmann, 1983). The availability in a synapse of both GABA and glycine could thus provide a more finely tuned control of the postsynaptic element than either GABA or glycine could exert alone. It is also possible that GABA and glycine interact at the receptor level: thus GABA has been shown in one system to modify allosterically the glycine receptor to increase its affinity for glycine (Werman, 1980). Glycine receptors occur postsynaptically to a proportion of the Golgi cell terminals, as shown at the ultrastructural level by means of specific antibodies against the receptor (Triller, Cluzeaud, and Korn, 1987)[2]. Antibodies against the GABA receptor (Schoch et al., 1985) have not yet provided results at a comparable level of resolution.

We have so far tacitly assumed that the glycine pool in the Golgi cell terminals participates in synaptic release. This would be in accord with data showing Ca^{++} dependent glycine release in cerebellar slices (Toggenburger, Wiklund, Henke, and Cuénod, 1983).

A coexistence of taurine and GABA in the Purkinje cell terminals is difficult to interpret. The nerve terminal pool of taurine might serve "metabolic" functions only (e.g., related to osmoregulation), although the higher concentration of taurine in the terminals than in the cell bodies does speak in favour of a role connected to synaptic release. The situation may be parallel to that of the co-existence of GABA and glycine (see above). In addition to its possible role as transmitter, taurine may modulate GABAergic transmission, e.g., by decreasing GABA release (Namima, Okamoto, and Sakai, 1983).

Conclusion

Using the cerebellum as a model we have presently shown that immunocytochemistry can be utilized to reveal the fine distribution of inhibitory amino acids in normal brain tissue. However, an equally important potential of this technique is its usefulness for investigating changes in amino acid localization under pathological and experimental conditions, e.g., after

[2]The glycine receptors were reported to be postsynaptic only to GAD *negative* Golgi terminals. This observation is difficult to reconcile with the present findings.

acute epileptic seizures (Meldrum, Swan, Ottersen, and Storm-Mathisen, 1987); or after depolarization or other manipulations of brain slices *in vitro* (Storm-Mathisen et al., 1986). These dynamic aspects of amino acid neurotransmission cannot be addressed by means of more indirect methods for transmitter localization, such as immunocytochemistry of transmitter synthesizing enzymes or autoradiography of transmitter uptake.

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