This uptake is inhibited by stilbene analogues which inhibit the chloride/bicarbonate antiport; these compounds also greatly inhibit the efflux of amino acids and the decreased glutamine synthesis seen with kainate administration. These compounds have no effect on veratridine-stimulated efflux of transmitter amino acids.

The question always arises in experiments \textit{in vitro} as to the relevance of the situation \textit{in vivo}. Rothman (1985) has shown that the neurotoxicity of glutamate, N-methyl-aspartate and kainate in hippocampal tissue cultures is associated with chloride influx; toxicity could be prevented by incubation in low chloride-containing medium. Olney et al. (1984) have made similar observations with chick retinal preparations. We have confirmed the latter study with chick retina and also found that inhibitors of chloride/bicarbonate exchange added to normal chloride medium greatly inhibit the efflux of amino acids and the decreased damage) now under active investigation in many laboratories.


Received 14 October 1986

\textbf{Tracing of neurons with glutamate or $\gamma$-aminobutyrate as putative transmitters}

where it can be detected by autoradiography, affording detailed information on the distribution of pathways (Vaughan & Gilbert, 1980; Streit, 1980; for review, see Cuénod \textit{et al.}, 1982). For this purpose it is necessary to use labelled $\Delta$-aspartate, a metabolically inert substrate for the Na$^+$-dependent, high-affinity acidic amino acid transporter (Davies & Johnston, 1976). In a recent modification of this technique (Fischer \textit{et al.}, 1986) the $\Delta$-[H]aspartate is implanted in solid form, absorbed in agarose particles packed into the tip of a glass capillary. This allows the tracer to be administered to a restricted area of brain and makes it possible to study even neurons with short axons. Although useful, the methods based on uptake may not be universally applicable, since neurons likely to use glutamate or aspartate as transmitter seem to vary in their ability to take up and axonally transport the labelled amino acid. In addition, particularly long axons may lose the tracer en route.

The physiologically most relevant marker for potentially glutamatergic nerve endings is the synaptic release process. Methods for studying Ca$^{2+}$-dependent depolarization-induced release of glutamate or aspartate from restricted regions (for review, see Abdul-Gahni \textit{et al.}, 1981) have yielded valuable information when the nerve endings responsible for the release have been identified by selective stimulation (Malthe-Sørenssen \textit{et al.}, 1979) or lesioning...
(Nadler et al., 1976; Hamberger et al., 1978) of nerve pathways. The observation experiments the endogenous contents of glutamate and/or aspartate are not as dramatically reduced in the pathway’s target area as are uptake or release (Fonnum, 1984), consistent with the fact that these amino acids are involved in general metabolism and hence are not restricted to the nerve endings where they may act as transmitters. Yet their concentrations may be higher than the tissue average in such nerve endings. The measurement of glutamate content in synaptosome preparations rather than in whole tissue samples of the target area of a lesioned nerve pathway may therefore be advantageous (Nadler & Smith, 1981).

The advent of immunocytochemical methods for localizing amino acids in glutaraldehyde-fixed tissue (Storm-Mathisen et al., 1983; see also Ottersen et al., 1986) now makes it possible to study the localization of glutamate and aspartate (Ottersen & Storm-Mathisen, 1985), as well as other related amino acids, at the level of resolution of the light and electron microscope. This approach has given particularly interesting results in cerebral slices incubated in different media (e.g. depolarizing media with or without Ca2+), precursors, enzyme inhibitors) before fixation by immersion in 5% glutaraldehyde. Such studies have shown that the hippocampal formation support the roles of glutamate and aspartate as transmitters in several of the excitatory neurons of this region, as well as the role of glia for the function of the nerve terminals (Storm-Mathisen et al., 1986a,b). They also show that experimentally induced changes of amino acid contents can indeed be studied in cells and nerve endings.

To approach more closely the situation in vivo, it appears best to investigate animals fixed by rapid perfusion with glutaraldehyde. However, in this type of preparation it has turned out to be difficult to display the ‘transmitter pool’ of glutamate (Ottersen & Storm-Mathisen, 1985; Storm-Mathisen & Ottersen, 1987). Part of the reason may be steric hindrance of the interaction of antibodies with fixed glutamate in the ‘transmitter pool’ of nerve terminals. This problem can be obviated by the post-embedding immunogold procedure which only displays antigens exposed on the surface of the ultrathin sections.

Electron microscopic visualization of glutamate-like immunoreactivity (Glu-LI) has been performed by means of this glutamate is indeed concentrated in synaptic vesicles and in cytosol (see Storm-Mathisen & Ottersen, 1987). A comparable high concentration of immunogold particles occurred over mitochondria in the same terminals. While it is conceivable that the apparent concentration of Glu-LI in vesicles and mitochondria relative to cytosol could be partly due to factors such as differences in conditions for fixation of glutamate between the compartments, the results provide strong circumstantial support for the view that glutamate is stored in vesicles independent of synaptosomes (Nadler et al., 1976; Hamberger et al., 1978).

A considerable number of excitatory nerve pathways in the central nervous system have been pointed out as putatively glutamatergic or aspartatergic by one or several of the approaches outlined above. The most conspicuous are the cortico-cortical and corticofugal pathways, the latter containing descending fibres from neocortical areas to the caudate nucleus, thalamus, pontine nuclei and spinal cord. Other putative glutamatergic pathways include several types of sensory neurons. In the cerebellum parallel and subpopulations of mossy fibres may be glutamatergic, whereas climbing fibres from oliva inferior may be aspartatergic. The references pertaining to the individual pathways have recently been tabulated (Ottersen & Storm-Mathisen, 1986a,b).

It should be emphasized that results obtained by the tracing methods need to be followed up by experiments on evoked synaptic release and on comparison of the effects of synaptically released transmitter and exogenously applied transmitter candidate, as well as of selective agonist and antagonist drugs. Finally, the fact should be kept in mind that other excitatory compounds apart from glutamate and aspartate may prove to be important transmitters in brain (e.g. french-Mullen et al., 1985; Cuénod et al., 1986).

Tracing of putative GABA-ergic neurons has been possible for many years. The selective localization of the GABA-synthesizing enzyme glutamate decarboxylase (GAD) in a type of neuron in brain was first demonstrated 16 years ago by lesion-induced loss of GAD activity from the target area of the axons of the inhibitory GABA-ergic neurons to the caudate nucleus (Fonnum et al., 1970). Tissue content of GABA in brain appear to be present in axon terminals. Biochemical evidence for the concentration of GABA and/or GAD in such neurons has been obtained by demonstrating losses after axonal sparing lesions induced by local injections of kainate and similar compounds (Coyle et al., 1978; Fonnum & Walaas, 1978).

Uptake of [1H]GABA or radiolabelled analogues in vitro or in vivo combined with autoradiography has proved suitable for tracing putative GABA-ergic neurons (Schon & Iversen, 1972; Hökfelt & Ljungdahl, 1970). Retrograde axonal transport probably contributes to the labelling of local neurons after intracerebral injections in vivo. However, this procedure is above all useful for tracing projection pathways (Streit, 1980).

After the purification of GAD and the raising of antibodies to it (see Wu et al., 1986), immunocytochemical visualization of GAD-containing perikarya and nerve endings became possible (Somogyi et al., 1987) showed that the putative glutamatergic nerve endings of mossy and parallel fibres contained about two times higher concentrations of Glu-LI (i.e. gold particle density) than putative GABA-ergic terminals (of Golgi cells), and about five times higher concentrations of Glu-LI than glial processes. Although the quantitative relationship between the grain density and the glutamate concentration in situ remains to be determined, these results show that glutamate is significantly concentrated in putative glutamatergic nerve endings.

Within the nerve endings of parallel fibres Glu-LI appeared to be five times more concentrated in synaptic vesicles than in cytosol (see Storm-Mathisen & Ottersen, 1987). A comparable high concentration of immunogold particles occurred over mitochondria in the same terminals. While it is conceivable that the apparent concentration of Glu-LI in vesicles and mitochondria relative to cytosol could be partly due to factors such as differences in conditions for fixation of glutamate between the compartments, the results provide strong circumstantial support for the view that glutamate is stored in vesicles independent of synaptosomes (Nadler et al., 1976; Hamberger et al., 1978).

Like other amino acids, GABA may be demonstrated in glutaraldehyde-fixed tissue by polyclonal (Storm-Mathisen et al., 1983; Ottersen & Storm-Mathisen, 1984a,b; Seguela et al., 1984; Hodgson et al., 1985) or monoclonal (Matute & Steit, 1987) antibodies. This method has the advantage of not being limited by species differences in GAD, and allows GABA to be detected independent of where and how it is produced. In addition, the antigen and similar antigens of related substances are easily available for testing of specificity (see Ottersen et al., 1986a).

For visualizing putative GABA-ergic neurons the GABA- and GAD-immunocytochemical methods produce generally similar results in mammalian brain and spinal cord. One case of apparent concentration of GAD has recently been expected to be found in the perikarya of the cerebellar Purkinje cell, the ‘classical’ GABA-ergic neuron. Whereas one GAD-antiserum readily demonstrates GAD-LI in these
cells (Oertel et al., 1981; Mugnaini & Oertel, 1985), GAD has been difficult to reveal in the perikarya of these cells by Wu et al. (1985) including a recovery of polyvalent and monoclonal antibodies (Wu et al., 1986). However, Somogyi et al. (1985) using an antisera from Wu et al. to demonstrate GAD-LI in the Purkinje cells (without blocking axonal transport by colchicine), although the staining was much stronger in the other cerebellar GABA-ergic cells. In the same material there was a very close correspondence between the immunoreactivities of GAD-LI and GABA-LI. The same pattern of staining is shown by two of our antisera, while our most used serum (26) gives poor staining of Purkinje cell bodies (Ottersen et al., 1984a). Interestingly, the chicken way and newly detected corticopetal pathways from septum to the deep cerebellar nuclei (as well as the terminals of basket cells surrounding the Purkinje cell perikarya) suggests that GABA-LI in the Purkinje cells than that the rat (Matute & Streit, 1987).

Most brain regions have been shown to contain GAD-rich or GABA-rich interneurons, from which it can be inferred that GABA-ergic inhibition is of general importance to the functioning of the central nervous system. Examples of GABA-ergic projection pathways include the cerebellar Purkinje cells, the striato-nigral pathway, and newly detected corticopetal pathways from septum and hypothalamus (for reviews, see Mugnaini & Oertel, 1985; Ottersen & Storm-Mathisen, 1984a). It should be noted that GABA-rich neurons have also been detected in the enteric nervous system (Davanger et al., 1986). As an example, the GABA content in hippocampus is increased after seizures induced by bicuculline (Chapman et al., 1984). We were able to show (Meldrum et al., 1987) that this increase is due to GABA-containing nerve terminals surrounding the pyramidal and granular cell perikarya, terminals thought to be particularly important for controlling the discharge of these neurons. On the other hand, GABA-containing nerve endings in the molecular layer of area dentata showed little change.

Finally, it should be recalled that GABA is not the only inhibitory amino acid transmitter, glycine being an important transmitter candidate, particularly in the spinal cord (Aprison & Nadl, 1978). We have recently demonstrated glycine-like immunoreactivity (Gly-LI) to be selectively localized in a physiologically, pharmacologically and morphologically characterized putative glycineergic neuron which controls motor neuron output in the Xenopus laevis spinal cord (Stell et al., 1986). Gly-LI-containing neurons also occur in mammalian spinal cord, brain stem, retina and cerebellum (Pourcho & Goebel, 1985; Campistron et al., 1986; Ottersen et al., 1987; Storm-Mathisen et al., 1986c).

This work was supported by the Norwegian Research Council for Science and the Humanities, the Norwegian Council on Cardiovascular Disease and the Norwegian Society for Fighting Cancer.


Localization and function of glutamine synthetase and glutaminase

GERD SVENNEBY and INGEBORG AASLAND TORGNER

Neurochemical Laboratory, Preclinical Medicine, University of Oslo, P.B. 1115 - Blindern, 0317 Oslo 3, Norway

Glutamine synthetase (GS) catalyses the reversible formation of glutamine from glutamate, ammonia and ATP (Levintow & Meister, 1955). A summary of the glutamine synthesis reaction is as follows. The enzyme possesses separate binding sites for glutamate, ATP and ammonia, but the substrates do not bind covalently to these sites. Ammonia binding requires prior binding to glutamate.

A divalent cation (Mg$^{2+}$, Mn$^{2+}$ or Co$^{2+}$) is required for activity of GS. $K_m$ for glutamate is 1.8 mM (Cooper et al., 1983). The inhibitor L-methionine-S-sulphoximine (MSO) binds to both the glutamate- and ammonia-binding sites. MSO is a strong irreversible inhibitor of GS, and administration of MSO to animals produces generalized convulsions (Cooper et al., 1983).

GS is mainly, if not exclusively, localized in the cytoplasm of astrocytes (Norenberg & Martinez-Hernandez, 1979). This discovery led to the suggestion that the astrocytes comprised a compartment in the brain engaged in the synthesis of glutamine. High concentrations of GS are also demonstrated in primary astrocyte cultures (Schousboe et al., 1975; Brandt et al., 1981) have demonstrated GS actively in these cultures by immunohistochemical studies. The immunohistochemical presence of GS in cultured astrocytes and its absence in oligodendrocytes have been reported by Raff et al. (1983).

The sheep brain enzyme has an apparent molecular mass of 392 kDa and is composed of eight identical subunits with molecular masses of approx. 64 kDa (Svenneby et al., 1973, 19866), except rat brain PAG, which consists of two different subunits with molecular masses of approx. 64 and 67 kDa (Svenneby et al., 1986b), respectively. Immunological studies have shown that the 67 kDa subunits account for about one-quarter of the total enzyme (Haser et al., 1985). The presence of apparent identical immunoreactivity in the above mentioned species reveals a rather conserved enzyme. Svenneby et al. (1986b) have demonstrated PAG structures (Svenneby et al., 19866) except rat brain PAG, whereas bovine brain PAG antibodies with higher titre. The dissociated subunit of PAG is enzymically inactive. The subunit can be dimerized to an active species, or aggregated to higher aggregates with molecular mass to up to about 2000 kDa (Svenneby, 1971). The higher aggregates increase in specific activity. The dimeric enzyme seems to have low or no activity in the absence of activator (e.g. phosphate), whereas the more aggregated forms are active in the absence of activator. Phosphate promotes dimerization of the enzyme, aggregation of the dimer enzyme form to tetrameric form, whereas borate in addition to phosphate enhances the aggregation to higher molecular mass species (Svenneby et al., 1973).

According to Svenneby et al. (1986b), only one inactivation of glutamine synthetase by phosphatase has been described (Svenneby et al., 1971; Chiu & Boecker, 1979; Nimmo & Tipton, 1981; Shapiro et al., 1982). Recently we have obtained evidence for two kinetically different PAG species in the mitochondria. One form is soluble or weakly associated with the membrane, and has a high $K_m$ (20-40 mM). The other form is tightly associated with the membrane, and the $K_m$ is much lower (about 2 mM). Both forms are competitively inhibited by both glutamate and ammonia. However, when kinetic studies are performed

by several investigators (for review, see Svenneby, 1984). PAG is shown to be an allosteric enzyme (Svenneby, 1971). A lot of di- and tri-carboxylic acids, in addition to phosphate and sulphate, activate the enzyme (O'Donovan & Lotspeich, 1966; Weil-Malherbe, 1969; Svenneby, 1971), as do calcium (Kvamme et al., 1983) and acyl-CoA derivatives (Kvamme & Torgner, 1984) (for review, see Svenneby, 1984; Kvamme et al., 1985). Glutamate, one of the reaction products, inhibits PAG, whereas both inhibition and activation by ammonia have been described. No significant difference concerning the kinetic properties is detected between the enzyme from either mammalian kidney or brain.

PAG is localized in the mitochondria, presumably in the inner mitochondrial membrane (for review see Svenneby, 1984). The brain enzyme from all mammals so far investigated, in addition to that of fish and bird, is built up of identical subunits with a molecular mass of approx. 64 kDa (Svenneby et al., 19866), except rat brain PAG, which consists of two different subunits with molecular masses of approx. 64 and 67 kDa (Svenneby et al., 1986b), respectively. Immunological studies have shown that the 67 kDa subunits account for about one-quarter of the total enzyme (Haser et al., 1985). The presence of apparent identical immunoreactivity in the above mentioned species reveals a rather conserved enzyme. Svenneby et al. (1986b) have demonstrated PAG structures (Svenneby et al., 19866) except rat brain PAG, whereas bovine brain PAG antibodies with higher titre. The dissociated subunit of PAG is enzymically inactive. The subunit can be dimerized to an active species, or aggregated to higher aggregates with molecular mass to up to about 2000 kDa (Svenneby, 1971). The higher aggregates increase in specific activity. The dimeric enzyme seems to have low or no activity in the absence of activator (e.g. phosphate), whereas the more aggregated forms are active in the absence of activator. Phosphate promotes dimerization of the enzyme, aggregation of the dimer enzyme form to tetrameric form, whereas borate in addition to phosphate enhances the aggregation to higher molecular mass species (Svenneby et al., 1973).

There seem to be some discrepancies between investigations concerning inhibition. Competitive, non-competitive and mixed competitive glutamate inhibition have all been described (Svenneby, 1971; Chiu & Boecker, 1979; Nimmo & Tipton, 1981; Shapiro et al., 1982). Recently we have obtained evidence for two kinetically different PAG species in the mitochondria. One form is soluble or weakly associated with the membrane, and has a high $K_m$ (20-40 mM). The other form is tightly associated with the membrane, and the $K_m$ is much lower (about 2 mM). Both forms are competitively inhibited by both glutamate and ammonia. However, when kinetic studies are performed

Abbreviations used: GS, glutamine synthetase; MSO, L-methionine-S-sulphoximine; PAG, phosphate-activated glutaminase, GABA, $\gamma$-aminobutyrate.