Selective accumulation of [^{3}H]GABA by stellate cells in rat cerebellar cortex *in vivo*

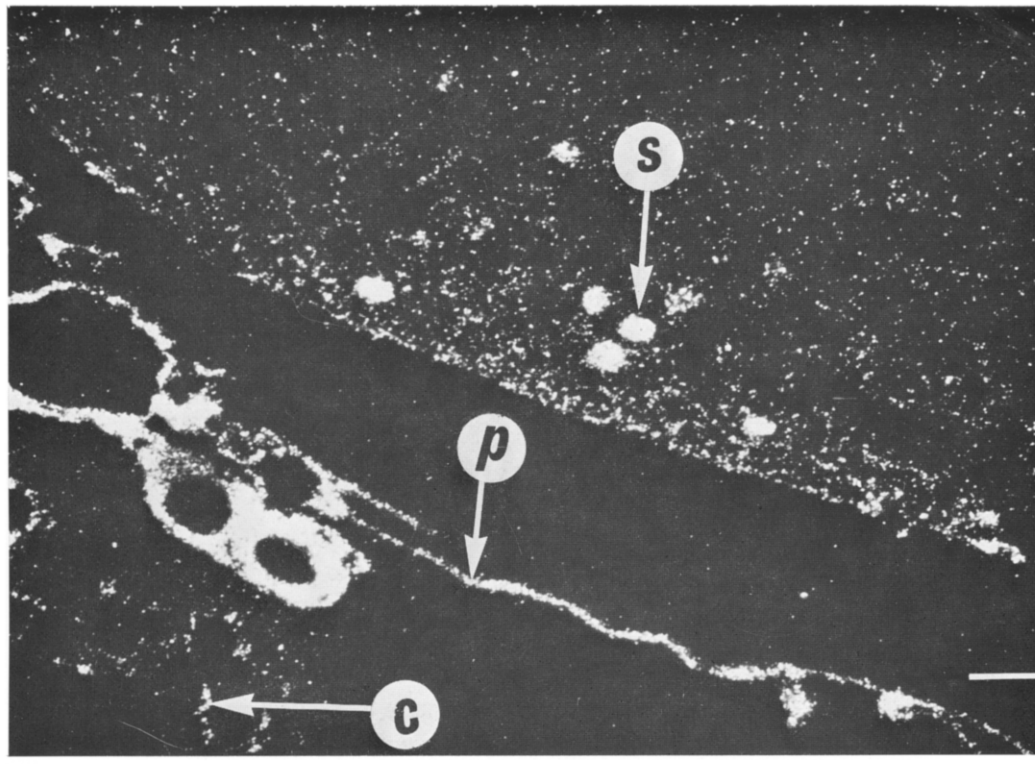
Autoradiographic studies of the localization of radioactively labelled gamma-aminobutyric acid (GABA) in mammalian brain slices and homogenates have indicated that this amino acid is preferentially taken up by nerve terminals or synaptosome particles[3,4]. There is evidence from biochemical studies that the labelled amino acid is taken up by the same nerve terminals which synthesize and store endogenous GABA in the brain[13]; consequently the autoradiographic localization of [^{3}H]GABA may offer a method for identifying those neurones which use GABA as a transmitter substance in the mammalian CNS[7,11]. A similarly selective uptake of labelled glycine by nerve terminals in the spinal cord has also been reported[9,12].

In the present study we have examined the localization of [^{3}H]GABA in rat brain after administering the labelled amino acid by injection into the cerebrospinal fluid *in vivo*. In this method the tissue may be fixed by vascular perfusion, and hence much better preservation of fine structure is obtained for electron microscopic autoradiography than is possible with *in vitro* labelling procedures[3,4,9,12]. To prevent the normally rapid metabolism of [^{3}H]GABA, animals were treated with amino-oxyacetic acid (AOAA) which inhibits the catabolic breakdown of GABA[19].

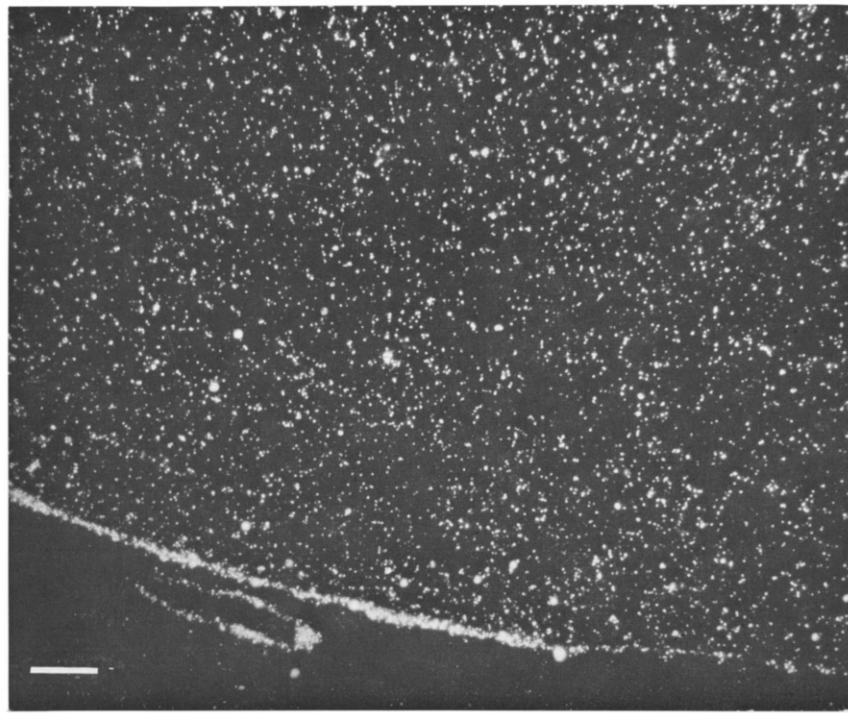
In preliminary experiments adult Wistar rats received a single intraperitoneal injection of AOAA (80 mg/kg) 30 min before injection of [^{3}H]GABA (2.5 μCi in 20 μl saline) into the lateral ventricle. The animals were killed 10 and 40 min after injection and were maintained under chloral hydrate anaesthesia throughout. The brains were homogenized in 0.2 *N* perchloric acid and centrifuged. The supernatant was passed through a column of Dowex 50 resin to remove any non-amino acid metabolites, and the amino acid fraction (eluted with ammonium hydroxide) was passed through a column of Dowex-1-acetate resin to remove any amino acids, according to the method of Iversen and Kravitz[10]. The purified material was examined by paper chromatography using 2 different solvent systems. The chromatograms were scanned for radioactivity, all of which appeared in a single peak corresponding in R_F to authentic GABA.

In animals treated with AOAA all of the radioactivity present in brain extracts was accounted for by unchanged [^{3}H]GABA. Ten minutes after injection, 27.7 $\pm$ 3.1% of the injected dose of [^{3}H]GABA remained in the brain, and there was no significant loss of labelled GABA between 10 and 40 min after injection. In animals not treated with AOAA, however, there was a smaller initial retention of [^{3}H]GABA (16.0 $\pm$ 1.4% at 10 min), followed by a rapid disappearance of the labelled amino acid from the brain. These values are means $\pm$ S.E.M. on groups of 4 animals.

For autoradiographic studies, AOAA pretreated rats were perfused with fixative 10 min after the injection of 100 μCi [^{3}H]GABA (spec. act. = 2.0 Ci/mmole) into the lateral ventricle. Brains were fixed by perfusion (through the heart into the aorta)[15] with a calcium containing buffered glutaraldehyde (5%) solution[16]. Preliminary experiments in which radioactivity was measured in brain tissue after such fixation indicated that approximately 50% of the [^{3}H]GABA was retained in the fixed tissue.



A



B

Fig. 1. A, Dark field autoradiograph of the floor of the cerebellum after [^{3}H]GABA administration as described in the text. Heavy accumulations of silver grains over superficial stellate cell bodies (s) and over the pia-arachnoid membrane (p) were observed. The choroid plexus (c) was relatively free of silver grains. B, The same region of the cerebellum after [^{3}H]glycine administration. Calibration bars = 7.3 μm.

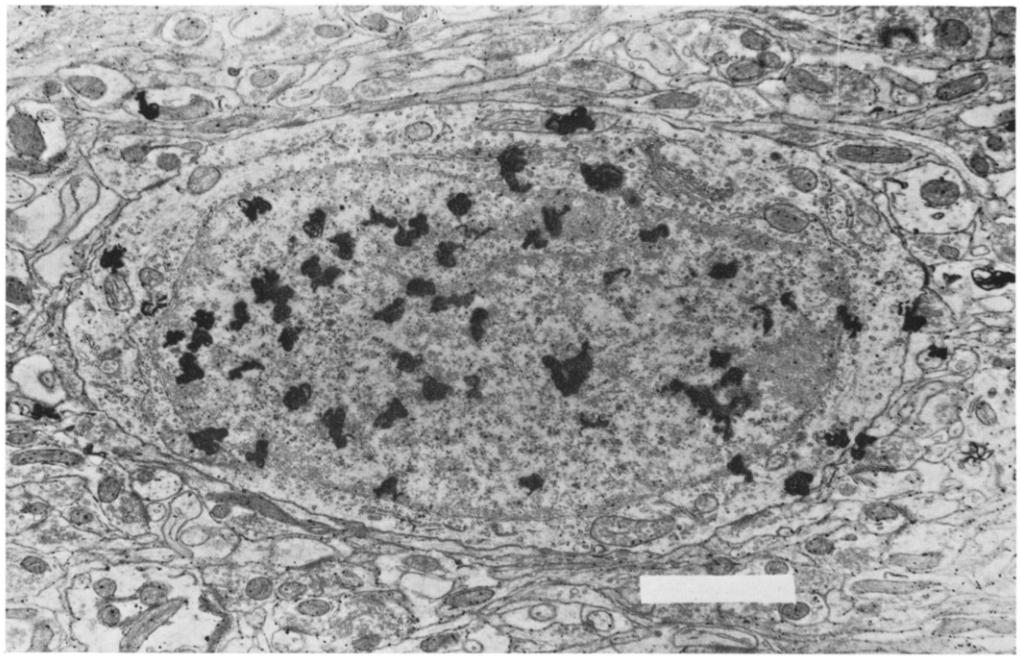


Fig. 2. Electron microscopic autoradiograph of the rat cerebellar cortex showing a heavy accumulation of silver grains over a superficial stellate cell. Calibration bar = 2.1 μm.

Various regions of the brain were dissected, treated with buffered osmic acid (1%), dehydrated and embedded in an epoxy resin. Thick sections (2 μm) were cut, mounted on glass slides and dipped in Ilford L4 emulsion for light microscope autoradiography. These sections were exposed in the dark for 1–6 weeks before developing.

Sections of the floor of the cerebellum overlying the IVth ventricle showed a dense accumulation of silver grains over certain superficial cell bodies (Fig. 1A). The pia-arachnoid membrane also showed a very high grain density, in contrast to the choroid plexus which was relatively free of grains. The labelled cell bodies in the cerebellar cortex were tentatively identified as stellate cells from a comparison of adjacent sections stained with methylene blue. Similar accumulations of [^{3}H]GABA over superficial cell bodies in the rat cerebellum have been observed using tissue slices incubated with [^{3}H]GABA[8].

Ultrathin sections (75 nm) were lead stained, carbon coated, dipped in emulsion and stored for 2–12 weeks before development and examination by electron microscopy. Prominent accumulations of silver grains were seen over superficial stellate cells (Fig. 2). These were identified by their position in the cerebellar cortex, their regular shape, the tongue shaped invagination of the nuclear membrane and the paucity of cristae in their mitochondria[6]. In Fig. 2 most of the silver grains lie over the cell nucleus. This observation was confirmed in most stellate cell bodies examined. The fixative action of glutaraldehyde probably involves the cross-linking of the amino group in the GABA molecule to an amino group in a side chain or N-terminal of a protein. Nucleoproteins such as histones contain a very high percentage of arginine and lysine

residues; the efficiency of glutaraldehyde cross-linkage formation may be, therefore, much higher in the nucleus than in the cytoplasm. Thus the observed distribution may not reflect the intracellular distribution of the GABA before fixation.

Stellate cell axons are thought to terminate on Purkinje cell dendrites[6]. These terminals did not consistently show a selective uptake of [^{3}H]GABA. This may be explained by the poor penetration of [^{3}H]GABA into the deep part of the cerebellar cortex which is where most of the stellate cell synapses are located. Most of the [^{3}H]-GABA is probably taken up into the pia-arachnoid membrane or the stellate cell bodies and their axons before it diffuses into the deeper layers of the cerebellum.

Evidence that this uptake of [^{3}H]GABA by stellate cells was specific, and not due to the activity of the neutral amino acid transport system present in all neurons[1], was obtained by comparing the former results with those obtained after injecting 100 μCi of [^{3}H]glycine into the lateral ventricle. The animals were killed after 10 min by aortic perfusion as before, and the same region of the cerebellum was dissected and prepared for light microscopic autoradiography. The results (Fig. 1B) showed an almost total absence of grain accumulations over stellate cells which were clearly discernible in phase contrast photographs of the same sections. There was however a diffuse labelling of the cerebellar tissue with [^{3}H]glycine, presumably as a result of the widespread distribution of the neutral amino acid transport system in the brain.

These findings support recent evidence that the pathway from superficial stellate cells onto Purkinje cell dendrites may involve GABA as an inhibitory transmitter. Electrophysiological evidence in the cat that the pathway is inhibitory is discussed by Anderson *et al.*[2]. Rushmer and Woodward[17] and Rushmer *et al.*[20] have also suggested that GABA is the transmitter in stellate cells of the frog cerebellum. They showed that both local surface stimulation (presumed to activate stellate cells) and iontophoretic application of GABA onto Purkinje cells caused a hyperpolarization which was blocked by bicuculline but not by strychnine.

There was also a prominent uptake of [^{3}H]GABA into the pia-arachnoid membrane in AOAA pretreated animals (Fig. 1A). This uptake was much reduced in animals not treated with AOAA, suggesting that GABA is normally metabolized rapidly after uptake in such extraneuronal sites. Histochemical studies have shown a high activity of GABA–glutamate transaminase in the pia-arachnoid membrane[18], which may represent part of the mechanism of the blood–brain barrier for GABA.

One restriction of the present method is the limited penetration of labelled amino acids from the ventricular system into the brain parenchyma. Thus this method can at present only be applied to structures situated close to the surface of the ventricular system. Preliminary observations in two such regions indicate neuronal localizations of [^{3}H]GABA. For example, in the locus coeruleus, which is known to contain mainly noradrenergic cell bodies[5,14], large numbers of nerve terminals making axodendritic synapses with the locus neurones were found to contain labelled GABA. Similarly in hypothalamic periventricular grey matter there were no labelled cell bodies but many labelled axodendritic synapses.

In conclusion, the present preliminary results suggest that autoradiographic studies after labelling with [^{3}H]GABA *in vivo* may provide a valuable new tool for

identifying transmitter-specific pathways in the CNS, although further studies are clearly needed to confirm the specificity of this method.

Department of Physiology and
M.R.C. Neurochemical Pharmacology Unit,
Department of Pharmacology,
University of Cambridge,
Cambridge (Great Britain)

F. SCHON
L. L. IVERSEN

1 ABANDOM, P. N., AND SCHOLEFIELD, P. G., Amino acid transport in brain cortex slices. II. Competition between amino acids, *Canad. J. Biochem.*, 40 (1962) 1591–1602.
2 ANDERSEN, P., ECCLES, J. C., AND VOORHOEVE, P. E., Postsynaptic inhibition of cerebellar Purkinje cells, *J. Neurophysiol.*, 27 (1964) 1138–1153.
3 BLOOM, F. E., AND IVERSEN, L. L., Localizing ^{3}H-GABA in nerve terminals of rat cerebral cortex by electron microscopic autoradiography, *Nature (Lond.)*, 229 (1970) 628–630.
4 BLOOM, F. E., AND IVERSEN, L. L., Studies of the uptake of ^{3}H-GABA and [^{3}H]glycine in slices and homogenates of rat brain and spinal cord by electron microscopic autoradiography, *Brain Research*, 41 (1972) 131–143.
5 DAHLSTRÖM, A., AND FUXE, K., A method for the demonstration of monoamine containing nerve fibres in the central nervous system, *Acta physiol. scand.*, 60 (1964) 293–294.
6 ECCLES, J. C., ITO, M., AND SZENTÁGOTHAI, J., *The Cerebellum as a Neuronal Machine*, Springer, Berlin, 1970, pp. 17–18.
7 HEBB, C., Identity of transmitter agents, *Ann. Rev. Physiol.*, 32 (1970) 165–192.
8 HÖKFELT, T., AND LJUNGDAHL, Å., Cellular localization of labelled gamma-aminobutyric acid (^{3}H-GABA) in rat cerebellar cortex: an autoradiographic study, *Brain Research*, 22 (1970) 391–393.
9 HÖKFELT, T., AND LJUNGDAHL, Å., Light and electron microscopic autoradiography on spinal cord slices after incubation with labelled glycine, *Brain Research*, 32 (1971) 189–194.
10 IVERSEN, L. L., AND KRAVITZ, E. A., The metabolism of γ-aminobutyric acid (GABA) in the lobster nervous system — uptake of GABA in nerve-muscle preparations, *J. Neurochem.*, 15 (1968) 609–620.
11 KRNJEVIC, K., Glutamate and γ-aminobutyric acid in brain, *Nature (Lond.)*, 228 (1970) 119–124.
12 MATUS, A. I., AND DENNISON, M. E., Autoradiographic localization of tritiated glycine at 'flat vesicle' synapses in spinal cord, *Brain Research*, 32 (1971) 195–197.
13 NEAL, M., AND IVERSEN, L. L., Subcellular distribution of endogenous and ^{3}H-GABA in rat cerebral cortex, *J. Neurochem.*, 16 (1969) 1245–1252.
14 OLSON, L., AND FUXE, K., On the projections from the locus coeruleus noradrenaline neurones: the cerebellar innervation, *Brain Research*, 28 (1971) 165–171.
15 PALAY, S. L., MCGEE-RUSSEL, S. M., GORDON, S., AND GRILLO, M. A., Fixation of neural tissues for electron microscopy by perfusion with solutions of osmium tetroxide, *J. Cell Biol.*, 12 (1962) 385–410.
16 PALAY, S. L., SOTELO, C., PETERS, A., AND ORKAND, P. M., The axon hillock and the initial segment, *J. Cell Biol.*, 38 (1968) 193–201.
17 RUSHMER, D. S., AND WOODWARD, D. J., Inhibition of Purkinje cells in the frog cerebellum. I. Evidence for a stellate cell inhibitory pathway, *Brain Research*, 33 (1971) 83–90.
18 VAN GELDER, N. M., A comparison of γ-aminobutyric acid metabolism in rabbit and mouse nervous tissue, *J. Neurochem.*, 12 (1965) 230–244.
19 WALLACH, D. P., Studies on the GABA pathway. I. The inhibition of γ-amino butyric acid–α-ketoglutaric acid transaminase *in vitro* and *in vivo* by N-7524 (amino oxyacetic acid), *Biochem. Pharmacol.*, 5 (1961) 323–331.
20 WOODWARD, D. J., HOFFER, B. J., SIGGINS, G. R., AND OLIVER, A. P., Inhibition of Purkinje cells in the frog cerebellum. II. Evidence for GABA as the inhibitory transmitter, *Brain Research*, 33 (1971) 91–100.

(Accepted April 14th, 1972)