Aspartate: Possible Neurotransmitter in Cerebellar Climbing Fibers

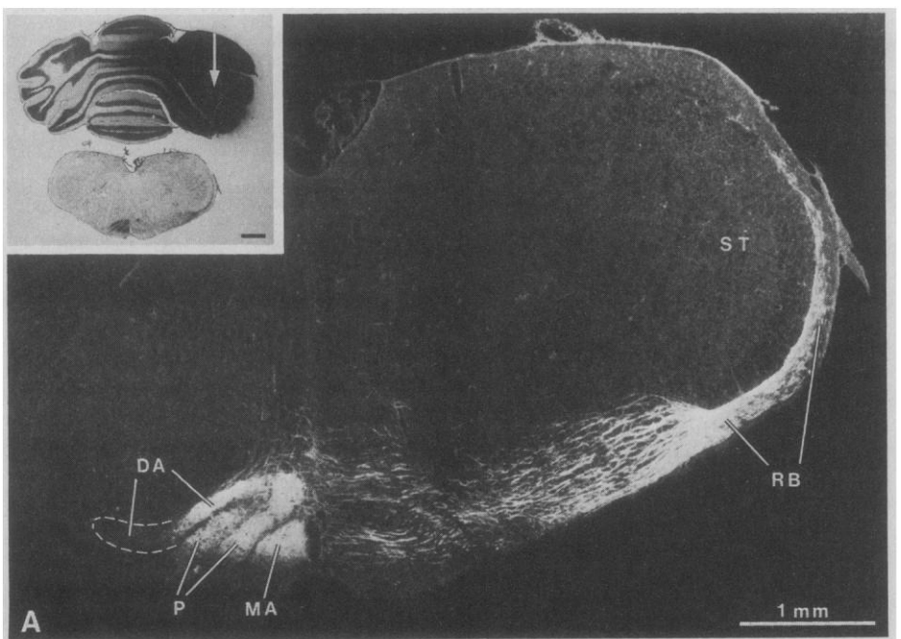
Abstract. *Autoradiography demonstrated prominent retrograde labeling of olivocerebellar climbing fiber neurons after injection of tritiated D-aspartate into the rat cerebellar cortex or deep nuclei. Mossy fiber systems originating in the brainstem and spinal cord remained unlabeled. Potassium ion–induced depolarization of cerebellar slices resulted in calcium ion–dependent release of endogenous L-aspartate, L-glutamate, γ-aminobutyric acid, and glycine. A 26 percent decrease in aspartate release was observed after 3-acetylpyridine-induced destruction of the inferior olive, supporting the hypothesis that aspartate is a neurotransmitter in climbing fibers.*

Excitatory afferents to the cerebellar cortex terminate as climbing or mossy fibers. The inferior olive is the origin of the climbing fibers (*1*, *2*), which synapse directly on the Purkinje cells (*1*, *3*). Mossy fibers arise from multiple sources in the brainstem and spinal cord (*4*) and synapse with the granule cells, excitatory interneurons contacting the Purkinje cells as parallel fibers (*1*, *3*). Little is know about the neurotransmitters active in these afferent systems (*5*), but excitatory amino acids are plausible candidates. Some studies have already implicated aspartate as a climbing fiber transmitter, based on a decrease of aspartate in cerebellar tissue after destruction of the climbing fiber system with 3-acetylpyridine (*6*). These studies, however, did not remain unchallenged (*7*). We report that olivocerebellar neurons are retrogradely labeled by D-[^{3}H]aspartate and that endogenous aspartate is released from these afferents.

Transmitter-specific retrograde labeling has been proposed as a way to identify the transmitter in some neuronal systems (*8*). To identify excitatory amino acids, retrograde labeling with D-[^{3}H]aspartate, an unnatural and metabolically inert enantiomer of aspartate, has been introduced (*9*, *10*). In this study, adult female SIV rats weighing 200 to 240 g were anesthetized with Nembutal and given microinjections of 50 nl of $10^{-2}M$ D-[^{3}H]aspartate (25 μCi, 10 to 18 Ci/mmole; New England Nuclear) stereotaxically into various parts of the cerebellar cortex (lobuli II to IX) and deep nuclei. Alternatively, vermis lobuli VI to VII were superfused for 2 hours with 10^{-5} or $10^{-4}M$ D-[^{3}H]aspartate in Krebs-Ringer solution. After 6, 12, or 24 hours the animals were perfused with 3.5 percent glutaraldehyde in 0.2*M* phosphate buffer (*p*H 7.4). Frozen sections of brainstem and spinal cord were then processed for autoradiography.

Intense retrograde labeling of the climbing fiber system was consistently observed after the injections into the cerebellum (Fig. 1). From the injection site, labeled nerve fibers could be traced through the underlying cerebellar white matter and restiform body and across the ventral medulla oblongata to their origin in the contralateral inferior olive, where labeled perikarya were observed in the regions known to project to the injected part of cerebellum (*11*). The large number of olivary perikarya labeled indicates that all olivary neurons share this capacity to accumulate D-[^{3}H]aspartate in their terminal region and to let the tracer migrate toward the cell bodies. In contrast, no mossy fiber sources in the brainstem or spinal cord were labeled. At the injection site, glial labeling was prominent. Granule cells were unlabeled, except for a small number that seemed to have been injured by the micropipette. The superfusions of vermis with D-[^{3}H]aspartate at concentrations in the range of the Michaelis constant for high-affinity uptake (*12*) also resulted in selective, albeit weaker, retrograde labeling of the olivocerebellar neurons.

These autoradiographic results indicate that the climbing fibers have a selective affinity for D-aspartate and that they may use an excitatory amino acid as a transmitter. Therefore, we investigated depolarization-induced release of endogenous amino acids from climbing fiber–deprived and control cerebellar slices.



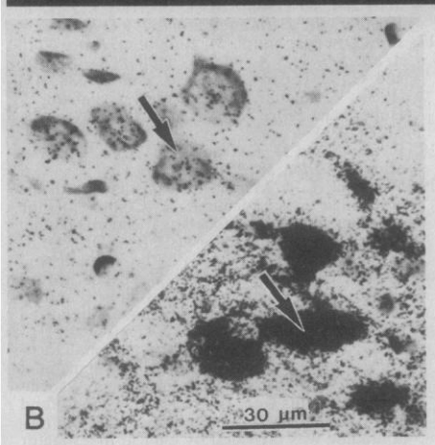


Fig. 1. (A) Dark-field micrograph of a section through the medulla oblongata of a rat that had received an injection of D-[^{3}H]aspartate into crus II and the paramedian lobule of the cerebellar hemisphere 24 hours previously. Retrogradely labeled nerve fibers are seen in the restiform body (*RB*) and traversing the ventral medulla oblongata from their origin in the contralateral inferior olive. At this rostral level of the inferior olive, strongly labeled perikarya are present in the medial accessory nucleus (*MA*), principal nucleus (*P*), and medial part of the dorsal accessory nucleus (*DA*). The lateral part of the dorsal accessory nucleus (outlined), which projects to the vermis of lobuli I to VI, is not labeled (*ST*, spinal trigeminal nucleus). Inset: micrograph showing injection site (arrow). The labeling in the inferior olive can easily be distinguished even at this low magnification. (B) Composite micrograph showing unlabeled neurons (arrow, upper field) in the dorsal accessory nucleus and intensely labeled neurons (arrow, lower field) in the principal nucleus. Increased neuropil labeling indicates tracer migration into the dendrites of labeled cells.

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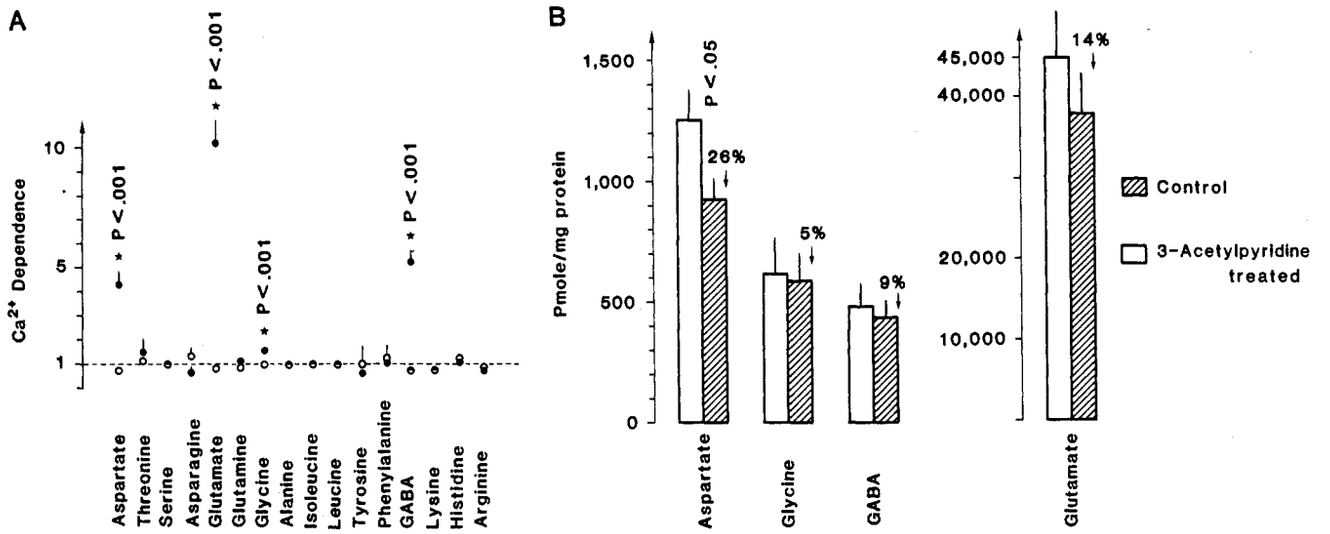

Fig. 2. Endogenous release of amino acids in rat cerebellar slices. (A) Amino acid efflux before (○) and during (●) stimulation with 50 m*M* K^+. Dependence on Ca^{2+} is given by the following ratio: efflux with 2 m*M* Ca^{2+}/efflux with 0.1 m*M* Ca^{2+} and 12 m*M* Mg^{2+}. (B) Potassium ion–induced release of aspartate, glycine, GABA, and glutamate in control rats (N = 24) and in rats whose climbing fibers were destroyed with 3-acetylpyridine (N = 22). Each value (mean ± standard error) represents the difference between the evoked (50 m*M* K^+) and basal (5 m*M* K^+) efflux.

Climbing fiber input to the cerebellum was destroyed by treating rats with 3-acetylpyridine (*13*). Control rats received saline. Histological examinations demonstrated that 99.3 percent of the inferior olivary neurons were destroyed in the rats given 3-acetylpyridine (*14*). Two weeks later all the animals were killed. Slices of the cerebellar hemispheres (excluding lobuli IX and X) were prepared with a McIlwain tissue chopper and superfused in mini chambers with Earle's buffered salt solution (*15*) at a constant flow of 520 μl/min. Samples of the eluate were immediately frozen, lyophilized, and redissolved in lithium citrate buffer. Their amino acid content was then determined with an automatic amino acid analyzer (Biotronik) having a Durrum DC-4A cation-exchange column and *O*-phthalaldehyde fluorescence detection. Amino acid release was evoked by elevating the K^+ concentration to 50 m*M* for 2.5-minute periods (*16*). Calcium dependence was determined by comparing release in the presence of a normal Ca^{2+} concentration (2.0 m*M*) with release in the presence of low Ca^{2+} (0.1 m*M*) and high Mg^{2+} (12 m*M*). Amino acid contents in eluates were expressed as picomoles per milligram of tissue protein (*17*).

The efflux of aspartate, glutamate, γ-aminobutyric acid (GABA), and glycine from control cerebellar tissue was significantly higher when the concentration of K^+ was elevated to 50 m*M* under normal Ca^{2+} than under conditions of low Ca^{2+} and high Mg^{2+}. The efflux of 12 other amino acids remained unchanged (Fig. 2A). It appears, therefore, that these four amino acids are released from nerve terminals upon depolarization. Indeed, GABA is considered the inhibitory transmitter of the Purkinje cells and of several interneurons in the cerebellar cortex (*18*), and glycine, also a putative inhibitory transmitter (*19*), was recently suggested to play a role in some cerebellar Golgi cells (*20*). Glutamate release has already been observed and related to the granule cells (*21*), but a previous study failed to demonstrate release of aspartate from cerebellar tissue (*22*).

The release of aspartate from control tissue amounted to 1258 ± 125 pmole/mg and decreased by 26 ± 12 percent ($P < .05$) after destruction of the climbing fiber input (Fig. 2B). This corresponded to a decrease in the Ca^{2+}-dependent part of the aspartate release. The large amount of glutamate released from normal tissue (44,690 ± 6,370 pmole/mg) did not change significantly after 3-acetylpyridine treatment. However, since a major part of the glutamate release is likely to originate from the parallel fibers (*23, 24*), it is possible that this large release could obscure a lesion-induced effect. Similarly, the release of GABA and glycine did not change significantly after 3-acetylpyridine treatment.

Large amounts of aspartate were still released after total destruction of the olivocerebellar neurons. This may be accounted for by the granule cells, which seem to release some aspartate in addition to glutamate (*25*) but which probably lack (or display very little) high affinity for excitatory amino acids (*24, 26*). These data, therefore, identify the climbing fibers as a source of (presumably) synaptic release of aspartate but do not exclude the possibility that they also release glutamate. Furthermore, the results demonstrate that all the olivocerebellar afferents are retrogradely labeled with D-[^{3}H]aspartate.

It seems manifest that retrograde labeling with D-[^{3}H]aspartate can delineate excitatory amino acid connections in neuronal systems. Such selective labeling has been demonstrated in cortical projections to the neostriatum, lateral geniculate body, and dorsal column nuclei in mammals, as well as in the avian retinotectal pathway (*8–10*). However, presynaptic neuronal uptake mechanisms seem to be involved in the labeling (*8*), and these are not believed to distinguish between L-glutamate, L-aspartate, and D-aspartate, at least not in the rat cerebral cortex (*12*). Therefore, it is likely that D-[^{3}H]aspartate labels neurons utilizing aspartate, glutamate, or both. This labeling technique may be most useful when combined with biochemical detection methods.

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15. The solution contained 154 m*M* Na^+, 5 m*M* K^+, 2 m*M* Ca^{2+}, 1 m*M* Mg^{2+}, 137.75 m*M* Cl^-, 20 m*M* HCO_3^-, 1.25 m*M* $H_2PO_4^-$, 1 m*M* SO_4^{2-}, and 10 m*M* glucose, and was gassed with 5 percent CO_2 and 95 percent O_2 at 37°C (*p*H 7.5).
16. Osmolality was kept constant by reducing the Na^+ concentration by the same amount.
17. At the end of the experiment the tissue slices were removed from the chambers, homogenized in 10 percent (weight to volume) trichloroacetic acid, and centrifuged. Protein content was then determined by the Biorad protein assay, with bovine serum albumin used as the standard.
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Extracellular Potassium Ions Mediate Specific Neuronal Interaction

Abstract. *The giant interneurons from the nerve system of the cockroach* Periplaneta americana *exhibit a peculiar reciprocal synaptic interaction. The synaptic potentials are not blocked by addition of 5 millimolar cobalt chloride and have an extrapolated reversal potential close to 0 millivolt. Hyperpolarizing current injected into one cell does not spread to the other. Intracellular injection of tetraethylammonium ions into one giant interneuron increases the duration of the action potential of the injected cell to 30 milliseconds and reduces the rise time and amplitude of the postsynaptic response recorded in the other giant interneuron. These results indicate that the interaction between the interneurons is not mediated by conventional chemical or electrotonic synapses. All evidence points to generation of the potentials by localized increases in extracellular potassium concentrations as a consequence of firing of one neuron.*

Chemical and electrotonic synapses are the bases for specific connections between excitable cells. The specificity of connections mediated by chemical synapses is a consequence of the specific chemical interaction between neurotransmitters released from the presynaptic terminal and receptors located on postsynaptic cells. In electrotonically coupled neurons, specific connections are provided by relatively large hydrophilic channels that directly connect cells (*1*). In the present report we describe a specific connection between identifiable interneurons which is probably mediated by potassium ions. The interaction is made possible by close membrane proximity in restricted and defined regions.

We described previously (*2*) a monosynaptic connection between two adjacent giant interneurons in the metathoracic ganglion (T_3) of the cockroach *Periplaneta americana*. This connection exhibits unusual characteristics (see Fig. 1). Intracellular injection of current into one such neuron, *a* (see Fig. 1A), generates an action potential (Fig. 1A, upper trace) that is followed by a synaptic potential recorded from an adjacent giant interneuron, *b*. Likewise, stimulation of *b* generates a similar synaptic potential in *a* (Fig. 1B). Thus, the interaction between these giant interneurons is reciprocal (giant-to-giant interneuron synaptic potential or GGSP). The GGSP appears after a delay of 1 msec and has a rise time of 1 to 1.5 msec and a long decay time (60 to 100 msec). The GGSP rise time is faster than the rise time of chemically mediated synaptic potentials (2.8 to 3 msec) recorded at the same place and generated in the same area. For example, see the arrow in Fig. 1B, where a spontaneous synaptic potential in interneuron *a* is recorded (*3*). To characterize the properties of the GGSP we studied the dependence of GGSP amplitude on transmembrane potential of the postsynaptic axon. In Fig. 1D, the first potential (arrow) is a GGSP evoked by intracellular stimulation of an adjacent giant interneuron, whereas the second potential, the result of a chemical synapse, is generated by extracellular stimulation of the contralateral thoracic connective. Although the GGSP amplitude is only slightly increased by hyperpolarization of the membrane, the chemically mediated postsynaptic potential is increased by more than twofold when the giant interneuron membrane is hyperpolarized by 20 mV (Fig. 1C). On the basis of this type of experiment we estimate that the reversal potential of the GGSP is between −10 and 0 mV, whereas postsynaptic potentials from other sources reverse between −65 to −57 mV (*4*). The GGSP also differs from other postsynaptic potentials evoked at T_3 by its insensitivity to the addition of 5 m*M* $CoCl_2$ to the physiological solution. All other evoked and spontaneous potentials are blocked by the addition of cobalt ions, but the GGSP is not. The only observed effect of 5 m*M* $CoCl_2$ on the GGSP is a reduction in its decay time (*5*).

Several features of the GGSP, namely, the relatively short latency and the insensitivity to cobalt ions in the bathing solution and to transmembrane potential, points strongly to the presence of electrotonic synapses (*1*). This possibility, however, was ruled out by direct experiments. Hyperpolarizing or depolarizing rectangle current pulses sufficient to produce a −40 mV to +20 mV shift in the membrane potential of the injected giant interneuron did not produce any change in the membrane potential of the adjacent interneuron. Since these results are not consistent with conventional chemically or electrotonically mediated transmission, we investigated the possibility that the GGSP is mediated by an increase in the extracellular potassium concentration following the genera-

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