A tight interaction between synaptotagmin and the Ca^{2+} channel might lead to docking of synaptic vesicles at active zones. The efficiency of synaptic transmission could be controlled by modulation of the binding of synaptic vesicles to the active zone.

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Involvement of the *N*-methyl D-aspartate (NMDA) Receptor in Synapse Elimination During Cerebellar Development

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In many instances, the establishment of highly specific neuronal connections during development results from the rearrangement of axonal projections through the trimming of exuberant collaterals or the elimination of functional synapses or both. Although the involvement of the *N*-methyl D-aspartate (NMDA) subtype of the glutamate receptor has been demonstrated in the shaping of axonal arbors, its participation in the process of selective stabilization of synapses remains an open issue. In this study, the effects of chronic in vivo application of D,L-2-amino-5-phosphonovaleric acid (D,L-APV), a selective antagonist of the NMDA receptor, on the synapse elimination process that takes place in the developing cerebellum of the rat have been analyzed. D,L-APV treatment prevented the regression of supernumerary climbing fiber synapses in 49 percent of the recorded Purkinje cells, while the inactive isomer L-APV was ineffective. Thus, activation of the NMDA receptor is a critical step in the regression of functional synapses during development.

Since its first description by Ramon y Cajal (*1*), synaptogenesis between cerebellar Purkinje cells and olivary climbing fibers has been considered one of the best models to study selective stabilization of synapses during central nervous system development (*2*). In the rat, each Purkinje cell is innervated by several climbing fibers during the first week after birth. Then, a massive elimination of most synapses occurs through the withdrawal of axonal collaterals (*3*, *4*) without substantial cell death of olivary neurons (*5*). The innervation of each Purkinje cell by only one climbing fiber, typical of the adult stage, is attained on postnatal day (PN) 14 to 15.

Multiple innervation of the Purkinje cells persists in adulthood when development has occurred in the absence of their second main input, that is, the granule cells, as in several mutant mice (*6*), in rats irradiated with x-rays (*7*), and in ferrets infected with feline panleucopenia virus (*8*). The mechanisms that prevent synapse elimination in these models are unknown. However, these observations indicate that regression of the polyinnervation during normal development requires granule cell input. Impulse activity from these cells could be the critical signal for regression because neural activity is known to regulate synapse elimination at the neuromuscular junction (*9*) and is a prominent factor in shaping neuronal connectivity in the central nervous system (*10*, *11*).

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The NMDA subtype of the glutamate receptor participates in activity-dependent synaptic modifications underlying learning and memory in the adult hippocampus (*12*). During development, NMDA receptors are involved in the segregation of eye-specific stripes (*13*) and in the pruning of retinal axon arbors (*14*) in the central nervous system of amphibians. Moreover, the segregation of retinogeniculate fibers in ferrets (*15*) as well as the formation of ocular dominance columns in kittens (*16*) seem also dependent on the activation of the NMDA receptor, although the interpretation of these results is currently debated (*17*).

In the cerebellum, a transient sensitivity of Purkinje and granule cell responses to NMDA occurs during the period of synapse elimination in rodents (*18*). As both climbing fiber and parallel fiber afferents likely use excitatory amino acid transmitters such as glutamate (*19*), these observations led us to investigate whether the activation of NMDA receptors is involved in cerebellar synapse elimination.

D,L-2-amino-5-phosphonovaleric acid (D,L-APV), a selective NMDA receptor antagonist, was delivered chronically from an implant of Elvax polymer (*13*, *20*) applied onto the surface of the posterior vermis in 4- to 5-day-old rats (PN 4 to 5) just before the period of synapse elimination. The implants remained in situ until the number of climbing fibers innervating each Purkinje cell was determined by in vivo intracellular recording at 18 to 31 days of age (PN 18 to 31) (Fig. 1). In each Purkinje cell, this number was estimated (*3*, *4*) from the stepwise increase in amplitude of the climbing fiber excitatory postsynaptic potentials (CF-EPSP) that occurred spontaneously or that were elicited by gradually

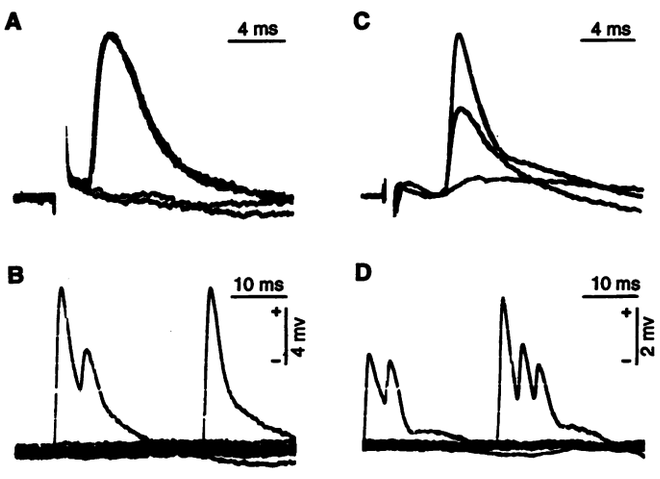

Fig. 1. Intracellular recordings from Purkinje cells of rats implanted with the NMDA antagonist D,L-APV. Climbing fiber EPSP occurring either spontaneously (**B** and **D**) or after electrical stimulation of the cerebellar afferents (**A** and **C**). Several sweeps are superimposed in all traces. The number of discrete steps provides a minimal estimate of the number of climbing fibers impinging on a Purkinje cell (*3*, *4*). Slow, sustained release of D,L-APV or its inactive stereoisomer L-APV was performed as described for amphibians (*13*). Sections (1 mm by 1 mm, 50 μm thick) of ethylenvinylacetate polymer (Elvax, DuPont) containing the drug (5 to 10 mM), (Sigma, St. Louis, Missouri) were implanted, after resection of the dura mater, on the surface of the posterior vermis of 4- to 5-day-old Wistar rats (PN 4 to 5) anesthetized with chloral hydrate; the animals were then allowed to recover until electrophysiological analysis. At PN 18 to 31, we obtained in vivo intracellular recordings from Purkinje cells in the posterior lobules of the vermis. Fast Green was iontophoretically deposited in two spots along the last electrode track for further reconstruction of the location of Purkinje cells. All cerebella were cut in a cryostat and stained with thionin.

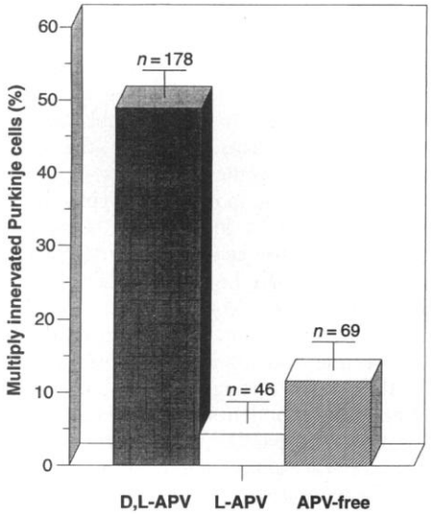

Fig. 2. Percentages of multiply innervated Purkinje cells recorded in the cerebella of D,L-APV, L-APV, and drug-free implanted rats (n = total numbers of Purkinje cells recorded in each group).

increasing the stimulation of the climbing fiber pathway.

Three groups of animals were studied: One group of 33 rats was treated with D,L-APV and two control groups of four and eight rats, respectively, were treated with the inactive stereoisomer L-APV or implanted with drug-free Elvax. The results (Fig. 2) indicate that, after D,L-APV treatment, 49% of the Purkinje cells abnormally retained multiple innervation, with a mean of 1.57 ± 0.64 (SD) climbing fibers per Purkinje cell (CF/PC) (n = 178). The control values were 4% with 1.04 ± 0.21 CF/PC (n = 46) after L-APV treatment and 12% with 1.16 ± 0.47 CF/PC (n = 69) in drug-free implanted animals. Significant differences were found only between D,L-APV–treated and each of the control groups, according to chi square test for percentage values and t test for mean values. The multiply innervated Purkinje cells retained two or three climbing fibers and were recorded in the superposed posterior folia as deep as 2000 μm under the implants. Regardless of the age, no differences were observed in any group.

The failure in the regression of supernumerary climbing fiber–Purkinje cell synapses is probably caused by the selective inhibition of the NMDA receptor by the D,L-APV treatment during the period when synaptic remodeling normally takes place. Indeed, in one animal the D,L-APV implant was asymmetrically located on the right hemivermis: All five Purkinje cells recorded in this region were polyinnervated, whereas all of the five cells recorded in the left hemivermis were monoinnervated. An insufficient amount of D-APV in the racemic mixture and differential susceptibility of the Purkinje cells could explain why only 49% of them retained polyinnervation. Except for their stepwise variation in amplitude, due to polyinnervation, the CF-EPSP did not exhibit any obvious difference in shape or frequency from that of the normal animal (Fig. 1). Similarly, the histological analysis did not show any sign of toxicity caused by this treatment (Fig. 3). The cerebellar region underneath the implant exhibited an apparently normal structure, with the Purkinje cell monolayer located between well-developed molecular and granular layers. In some cases, however, slight abnormalities of foliation were observed in the posterior cerebellum of D,L-APV–treated and control rats as well, presumably due to the surgery. But even in these cases, the granular and the molecular layers around the recorded Purkinje cells displayed a normal morphology under the light microscope. Granule cell density at 3 to 4 weeks of age was decreased slightly in the D,L-APV group but was decreased even more in the L-APV rats (Table 1). Given the monoinnervated condition of L-APV animals, such a limited granule cell loss (~10%) in D,L-APV rats is therefore unlikely to account for the persistence of the multiple innervation. A toxic effect of L-APV treatment suggested by the resulting slight but significant cell loss could be related to a reported excitatory effect of L-isomers of aminodicarboxylic amino acids (*21*), similar to the well-known excitotoxic effects of glutamate receptor agonists.

Previous reports have pointed to a pivotal role for NMDA receptor activity in the refinement and pruning of axonal arbors (*13–15*) and in the establishment of functional properties (*16*, *17*) in the developing visual system. However, it is still unclear whether these processes are associated with synapse elimination between retinal afferents and their postsynaptic targets. Furthermore, data on another example of axonal exuberancy, that is, the transient transcallosal connections between visual cortices in kittens, suggest that the supernumerary ax-

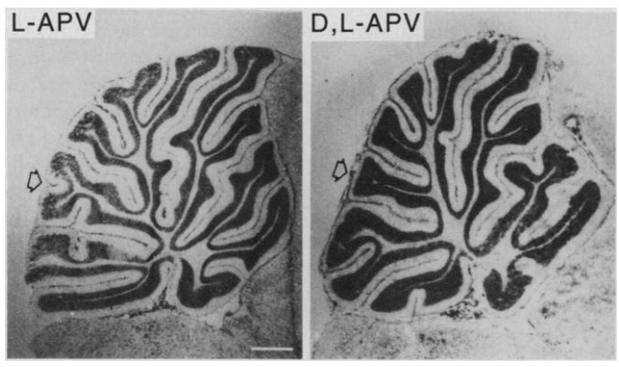

Fig. 3. Representative sagittal cryostat sections of cerebellar vermis of PN 18 rats treated with D,L-APV (right) and with the inactive stereoisomer L-APV (left). Arrowhead indicates the position of the implant under which the Purkinje cells have been recorded. Thionin staining. Bar, 1 mm.

Table 1. Estimate of granule cell density in the posterior cerebellar vermis of 3- to 4-week-old rats after chronic treatment with D,L-APV, L-APV, and APV-free implants. The granule cells were counted in 16 areas (1600 μm^2 each) of the granular layer selected randomly in one thionin-stained cryostat sagittal section per animal. Statistical significance ($P < 0.01$) was assessed by one-way analysis of variance. Significant differences in average density were found between the three groups; the granule cell loss was the most pronounced in the L-APV–treated cerebella where only a few multiply innervated Purkinje cells have been recorded.

Treatment	Granule cells per square millimeter	Number of animals
D,L-APV	39,775 ± 1,317	20
L-APV	35,086 ± 1,764	5
Drug-free	44,174 ± 2,451	7

ons regress but without ever establishing functional synapses (*22*). Our data, from intracellular recording from the postsynaptic target in a well-defined neuronal circuit, show that activation of the NMDA receptor participates in a process of true elimination of functional synapses in the developing cerebellum.

Although there is agreement on the transient hyperexpression of the NMDA receptor on the developing granule cells (PN 5 to 20), its transient presence on Purkinje cells is still controversial (*23*). If present on the Purkinje cells, the NMDA receptor could be activated by the depolarization induced by the parallel fiber synapses on the Purkinje cells and the concomitant binding of the excitatory amino acid transmitter from the climbing fiber afferents (or vice versa) (*24*). Through an increase of intracellular calcium concentrations, the NMDA receptor activation could trigger a cascade of biochemical events (*25*) leading to a selective stabilization of coactive synapses (*26*). In this way, the NMDA receptor could mediate the action of the granule cell pathway on climbing fiber–Purkinje cell synapse elimination. Alternatively, the possibility exists that NMDA receptors are absent from Purkinje cells but present on the granule cells (*23*); NMDA receptors have been identified at the mossy fiber–granule cell synapse (*27*) and at the parallel fiber–inhibitory interneuron synapse (*28*). D,L-APV could have modified the pattern of electrical activity conveyed by the granule cell input to the Purkinje cell either directly or through the inhibitory interneurons. In this case, an involvement of the NMDA receptor in synapse elimination would be indirect, acting through a modulation of afferent electrical activity. Direct effects on Purkinje cells and indirect action on granule cells are not mutually exclusive, and further studies would help to delineate their relative contribution.

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