Selective Activation of Calcium Permeability by Aspartate in Purkinje Cells

Michisuke Yuzaki,* Douglas Forrest, Tom Curran, John A. Connor

Glutamate and aspartate are endogenous excitatory amino acid neurotransmitters widely distributed in the mammalian central nervous system. Aspartate was shown to induce a large membrane current sensitive to *N*-methyl-D-aspartate (NMDA) and non-NMDA receptor antagonists in Purkinje cells from mice lacking functional NMDA receptors ($NR1^{-/-}$). This response was accompanied by high permeability to calcium. In contrast, no current was induced by aspartate in hippocampal neurons and cerebellar granule cells from $NR1^{-/-}$ mice. Several other glutamate receptor agonists failed to evoke this response. Thus, in Purkinje cells, aspartate activates a distinct response capable of contributing to synaptic plasticity through calcium permeability.

L-Glutamate (Glu) activates both NMDA and non-NMDA receptors, whereas L-aspartate (Asp) is a selective agonist for NMDA receptors because the currents produced by Asp (at a concentration of ≤5 mM) are completely blocked by NMDA receptor antagonists such as 7-Cl-kynurenic acid (7-Cl-Kyn) and Mg^{2+} (*1*). However, it is difficult to rule out the possibility that there are independent Asp-specific receptors that are blocked by NMDA receptor antagonists. To test this hypothesis, we analyzed neurons from $NR1^{-/-}$ mice that lack functional NMDA receptors (*2*). Thus, if there was a distinct receptor for Asp, Asp would be predicted to activate currents in $NR1^{-/-}$ neurons.

In voltage-clamped (−60 mV) hippocampal neurons and cerebellar granule cells from wild-type mice, NMDA (100 μM), Asp (100 μM), and Glu (100 μM) induced inward currents (Fig. 1A). In the corresponding neurons from $NR1^{-/-}$ mice, NMDA evoked no responses (Fig. 1B) (*2*). Similarly, no responses to Asp were detected in these neurons, consistent with reports indicating that Asp is a selective NMDA receptor agonist (*1*). This finding suggests that there are no distinct receptors for Asp in these neurons. The residual currents induced by Glu in $NR1^{-/-}$ neurons were mediated by non-NMDA receptors (Fig. 1B).

In Purkinje cells from wild-type mice, NMDA induced atypical transient currents (Fig. 1A). In contrast to the NMDA-induced currents in hippocampal and cerebellar granule cells, peak currents in Purkinje cells declined to zero during the steady application of NMDA. Notably, in $NR1^{-/-}$ Purkinje cells, NMDA induced atypical transient currents (Fig. 1B). Moreover, Asp evoked a substantial current in $NR1^{-/-}$ Purkinje cells (Fig. 1B), indicating that Purkinje cells express distinct receptors sensitive to both Asp and NMDA in the absence of NR1.

To determine whether the atypical responses to NMDA and Asp in $NR1^{-/-}$ Purkinje cells were an indirect consequence of the gene knockout, we characterized the pharmacological properties of the Asp responses in wild-type neurons. Asp-induced currents in hippocampal neurons were completely blocked by NMDA antagonists D,L-2-amino-5-phosphonovaleric acid (APV, 200 μM), Mg^{2+} (1 mM), and 7-Cl-Kyn (5 μM), but they were insensitive to the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) (Fig. 1C). The Asp-induced current was reduced by Mg^{2+} in a voltage-dependent manner (Fig. 1D). Dose-response analysis by means of a logistic equation showed a median effective concentration (EC_{50}) of 10.3 μM and a Hill coefficient of 1.5 (Fig. 1E), consistent with the view that the classical NMDA receptor is selectively activated by Asp in hippocampal neurons (*1*). In contrast, in wild-type Purkinje cells, Asp-evoked currents were blocked by CNQX and were moderately reduced by APV, Mg^{2+}, and 7-Cl-Kyn (Fig. 1C). Moreover, the blockade by Mg^{2+} was not voltage-dependent (Fig. 1D). Dose-response analysis

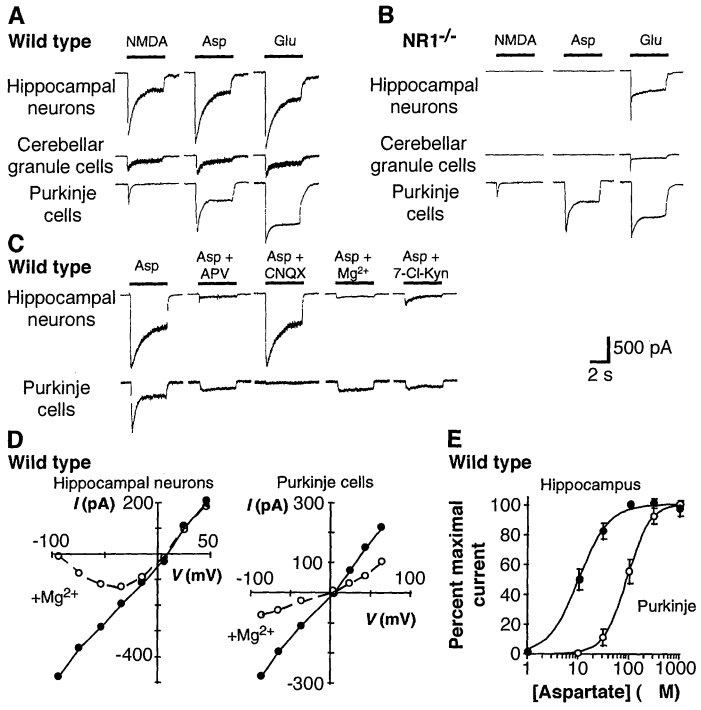


Fig. 1. Responses and pharmacology of neurons from wild-type and NR1 knockout mice to excitatory amino acids. Primary cultures were prepared from neonatal mice within 3 to 4 hours of birth as described (*2, 5, 23*). Membrane currents were measured by whole-cell patch-clamp methods at −60 mV after 7 to 18 days in vitro (*24*). (**A** and **B**) NMDA (100 μM), Asp (300 μM), and Glu (100 μM) were applied in Mg^{2+}-free, glycine (1 μM)-containing solutions to each type of neuron from wild-type (A) and NR1 knockout mice (B). Each trace represents a recording from 7 to 12 neurons. (**C**) Sensitivity of Asp-induced currents in wild-type neurons to glutamate receptor antagonists. Asp (300 μM) + glycine (1 μM) were applied without antagonists or in the presence of APV (200 μM), CNQX (10 μM) (*25*), Mg^{2+} (1 mM), or 7-Cl-Kyn (5 μM). APV, CNQX, Mg^{2+}, and 7-Cl-Kyn reversibly reduced the Asp-induced peak currents to 18 ± 2%, 1.6 ± 0.3%, 20 ± 2%, and 13 ± 0.6% of peak currents (mean ± SEM, $n = 7$), respectively. (**D**) Voltage dependence of block by Mg^{2+} (1 mM) of Asp (300 μM)-activated current in wild-type neurons. Peak currents were plotted against the holding potential of neurons. The graph represents the results from three to four neurons. (**E**) Mean dose-response curves for Asp in wild-type neurons. For each cell, the data were normalized to the current evoked by a saturating dose of Asp. Points indicate the mean ± SEM of the peak current amplitude values from seven to eight neurons; the curves indicate the best fits to the data according to the logistical function $1/\{1 + (EC_{50}/[Asp]^{n_H}\}$, where EC_{50} is the concentration causing a 50% maximal response and n_H is the Hill coefficient.

M. Yuzaki and T. Curran, Department of Developmental Neurobiology, St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38105-2794, USA. D. Forrest, Department of Human Genetics, Mount Sinai School of Medicine, Fifth Avenue at 100th Street, New York, NY 10029, USA. J. A. Connor, Lovelace Institutes, 2425 Ridgecrest Drive Southeast, Albuquerque, NM 87108, USA.

* To whom correspondence should be addressed. E-mail: michi.yuzaki@stjude.org

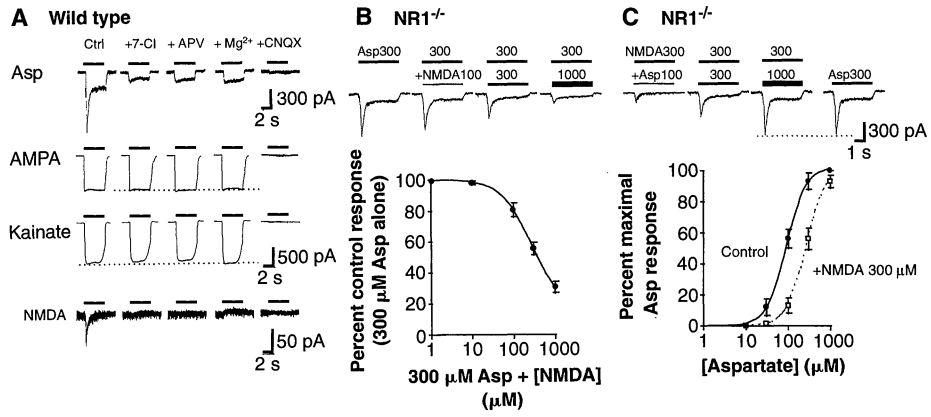
showed an EC_{50} of 91.4 μM and a Hill coefficient of 1.9 (Fig. 1E). This pharmacological profile of the Asp response was identical to that determined in $NR1^{-/-}$ Purkinje cells (Fig. 2D). Therefore, the specific Asp-induced response, which we refer to as the Asp response, was present both in wild-type and $NR1^{-/-}$ Purkinje cells (*3*).

We further characterized the Asp response by using known agonists of different classes of glutamate receptor. In the event that an agonist evokes a response from the putative Asp receptor, the current should be at least partly sensitive to antagonists of both NMDA and non-NMDA receptors, in a manner similar to that of the Asp-induced current in Purkinje cells (Fig. 2A). However, the currents activated by selective non-NMDA receptor agonists such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate were not blocked by NMDA receptor antagonists (Fig. 2A), indicating that AMPA and kainate were not effective agonists for the putative Asp receptors in Purkinje cells. In contrast, transient NMDA responses in Purkinje cells were blocked by the non-NMDA receptor antagonist CNQX in addition to classical NMDA receptor antagonists (Fig. 2A), suggesting that NMDA did activate the Asp response.

To exclude the possible involvement of classical NMDA receptors that have been shown to be expressed in immature Purkinje cells (*4–6*), we used $NR1^{-/-}$ Purkinje cells to further characterize the interaction of NMDA with the putative Asp receptor. When NMDA was coapplied with Asp to $NR1^{-/-}$ Purkinje cells, it reduced the Asp-induced currents in a dose-dependent manner (Fig. 2B) with a median inhibition concentration (IC_{50}) of 265 μM and a Hill coefficient of 1.2. When a fixed concentration of NMDA (300 μM) was used against increasing concentrations of Asp, NMDA appeared to act as a competitive inhibitor of the Asp response (Fig. 2C). Thus, the inhibition was most likely due to the competitive action of NMDA at Asp recognition sites. Thus, NMDA may be considered as a partial agonist, which binds to the putative receptor but does not fully activate the response, and which acts as an antagonist when applied in combination with Asp. The antagonism of Asp-induced voltage changes by *N*-methyl-D,L-Asp (NMDLA) observed in studies with Purkinje cells in slice preparations (*7*, *8*) may be due to this partial agonist action of NMDA.

The endogenous neurotransmitters Glu and homocysteate (*9*) can activate both NMDA and non-NMDA receptors. Thus, it is difficult to determine whether these agonists activate the Asp response in wild-type Purkinje cells. However, if these agonists evoke an Asp response, the currents should be sensitive to NMDA receptor antagonists in $NR1^{-/-}$ Purkinje cells, as observed for the Asp-induced current (Fig. 2D). However, the currents evoked by Glu and homocysteate were not blocked by NMDA receptor antagonists in $NR1^{-/-}$ Purkinje cells (Fig. 2D), indicating that Glu and homocysteate are not effective agonists for the putative Asp receptor.

To determine whether Ca^{2+} ions mediate the current induced by Asp, we mea-



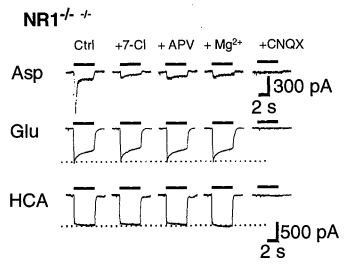


Fig. 2. Agonists for the putative Asp receptor. (**A**) Tests for agonists in wild-type Purkinje cells. Asp (300 μM), AMPA (20 μM), kainate (20 μM), or NMDA (300 μM) were applied in the absence (control) or presence of 7-Cl-Kyn (5 μM), APV (200 μM), Mg^{2+} (1 mM), and CNQX (10 μM). Representative traces are shown (n = 3 to 6). (**B** and **C**) Interaction of NMDA with the putative Asp receptor in $NR1^{-/-}$ Purkinje cells. (B) Asp (300 μM) + glycine (1 μM) were applied with various concentrations of NMDA as indicated. The graph shows the dose-response for inhibition by NMDA of the currents evoked by 300 μM Asp. Points indicate the mean ± SEM of the peak current amplitude expressed as a percentage of the Asp control response (n = 4 to 5); the curves indicate the best fits to the data according to the logistical function $1 - 1/(1 + IC_{50}/[NMDA]^{n_H})$, where IC_{50} is the concentration causing 50% maximal inhibition and n_H is the Hill coefficient. (C) Various concentrations of Asp + glycine (1 μM) were applied in the presence or absence of NMDA (300 μM). The graph shows the effect of 300 μM NMDA on the Asp-induced dose-response curve. Points indicate the mean ± SEM of the peak current amplitude expressed as a percentage of the current evoked by a saturating concentration of Asp (1 mM) (n = 4 to 5); the curves indicate the best fits to the data according to the logistical function $1/\{1 + (EC_{50}/[Asp]^{n_H})\}$ for control (solid line) and to the logistical function with competitive inhibition $1/\{1 + EC_{50}/[Asp]^{n_H} \times (1 + [NMDA]^{n_H}/K_i^{n_H})\}$, where K_i is the apparent antagonist affinity constant (dotted line). The EC_{50} was 88.2 μM with n_H = 1.9, and K_i was 104.3 μM. (**D**) Test for endogenous EAA agonists in $NR1^{-/-}$ Purkinje cells. Asp (300 μM), Glu (100 to 1000 μM), or homocysteate (100 to 500 μM) were applied in the absence (control) or presence of 7-Cl-Kyn (5 μM), APV (200 μM), Mg^{2+} (1 mM), and CNQX (10 μM). All solutions contained glycine (1 μM). Traces shown were obtained with 100 μM Glu (middle trace) and 100 μM homocysteate (bottom trace) (n = 5 to 7).

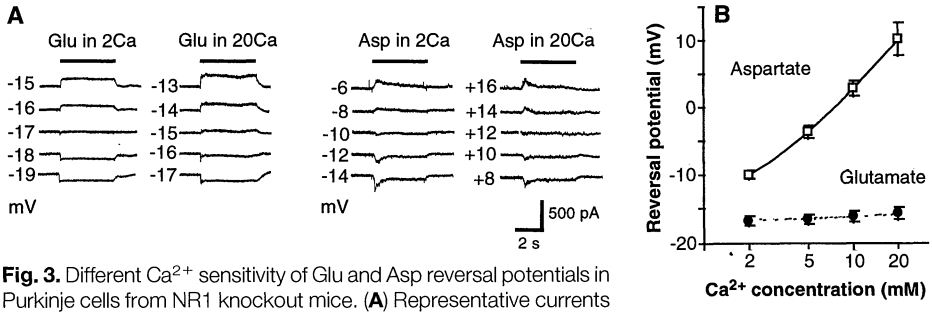


Fig. 3. Different Ca^{2+} sensitivity of Glu and Asp reversal potentials in Purkinje cells from NR1 knockout mice. (**A**) Representative currents induced by Glu (100 μM) + glycine (1 μM) (left traces) and Asp (100 μM) + glycine (1 μM) (right traces) in solutions containing 80 mM Na^+ and either 2 mM Ca^{2+} (2Ca) or 20 mM Ca^{2+} (20Ca) (*26*). (**B**) Reversal potentials for Asp (□) and Glu (●) as a function of external Ca^{2+} concentration. Each point represents the mean ± SEM of five to seven separate experiments (*27*). The lines were drawn according to the constant-field equation (*10*) with a nonlinear least-squares fit. Ionic activities were calculated with the empirical formula (*28*) modified for mixed electrolyte solutions and were used instead of ionic concentrations.

sured the reversal potentials of Asp-evoked currents in NR1$^{-/-}$ Purkinje cells while the external Ca^{2+} concentration was systematically changed (Fig. 3A). To quantitate the relative permeability of Ca^{2+} versus that of monovalent ions (P_{Ca}/P_{mono}), we used the Goldman-Hodgkin-Katz equation modified to include divalent cations, assuming no anion permeability and equal permeability for monovalent cations (*10*). This analysis yielded P_{Ca}/P_{mono} = 0.17 for Glu and 13.2 for Asp (Fig. 3B). The Ca^{2+} permeability of the Glu-activated channel is consistent with the reported value of the low–Ca^{2+}-permeable type of non-NMDA receptors (P_{Ca}/P_{mono} = 0.07 to 0.18) in many types of neurons (*10–12*). In contrast, the Ca^{2+} permeability of the Asp-activated response was much higher than the reported value of the high–Ca^{2+}-permeable type of non-NMDA receptors (P_{Ca}/P_{mono} = 1.1 to 2.3) (*11–14*) and comparable to that of NMDA receptors (P_{Ca}/P_{mono} = 10.6 to 14.3) (*10, 12*). This finding reinforces the view that Glu was not an effective agonist for Asp responses. Moreover, it rules out the possibility of indirect activation of other glutamate receptors resulting from the release of Glu by reversed Glu uptake (*15*) or heteroexchange (*16*), confirming the specific nature of the Asp response.

Molecules responsible for the Asp response have yet to be identified. Although it is possible that the response is mediated by an electrogenic excitatory amino acid (EAA) transporter, the characteristics of the Asp response are distinct from those of the known transporters. All known EAA transporters transport Glu as well as Asp, whereas the Asp response could not be evoked by Glu (Figs. 2D and 3). Moreover, the Asp response was sensitive to conventional glutamate receptor modulators (Fig. 2A) (*18*). Thus, it is more likely mediated by a distinct receptor-channel complex similar to conventional glutamate receptor channels. Alternatively, it could be mediated by an unidentified EAA transporter (*17*) that shares similar pharmacology with glutamate receptor channels.

Intracellular Ca^{2+} increases have been shown to provide critical functions in the establishment of long-lasting synaptic plasticity, such as long-term depression (*19*), and in excitotoxic cell death (*20*) in Purkinje cells. Thus, it is possible that the Asp response participates in these important functions through its high Ca^{2+} permeability. Asp has been shown to be contained in and released from climbing fiber terminals (*16, 21*) that make synapses onto Purkinje cells. However, the role of the Asp response in synaptic transmission is still disputed (*22*), partly because of the lack of pharmacological information on Asp responses in Purkinje cells. Here we have defined a specific pharmacology of the Asp response that should prove useful for its further characterization in physiological functions.

REFERENCES AND NOTES

1. D. K. Patneau and M. L. Mayer, *J. Neurosci.* **10**, 2385 (1990).
2. D. Forrest *et al.*, *Neuron* **13**, 325 (1994).
3. The Asp response may have consisted of two components with different kinetics, because the inactivating component of the response was more sensitive to NMDA receptor antagonists than was the noninactivating component (Fig. 1C). Furthermore, it exhibited a higher affinity for Asp (EC_{50} = 91 μM) (Fig. 1E) than did the noninactivating component (EC_{50} = 233 μM). In addition, although the inactivating component often diminished during prolonged recordings (> 30 min), there was little change in the noninactivating component (17 of 29 prolonged recordings). The presence of two components may account for the insensitivity of Asp currents to APV [E. Audinat, T. Knöpfel, B. H. Gähwiler, *J. Physiol. (London)* **430**, 297 (1990)]; that is, if the method of agonist application was slow, as is generally the case in slice preparations, the inactivating component is never fully expressed, leaving only the noninactivating component that we find to be less sensitive to NMDA receptor antagonists. The two components could be a consequence of several conducting states of a single molecule; alternatively, they could reflect the involvement of several different molecules.
4. C. Rosenmund, P. Legendre, G. L. Westbrook, *J. Neurophysiol.* **68**, 1901 (1992).
5. M. Yuzaki and K. Mikoshiba, *J. Neurosci.* **12**, 4253 (1992).
6. M. Yuzaki *et al.*, *ibid.* **16**, 6451 (1996).
7. F. Crepel, J.-L. Dupont, R. Gardette, *Brain Res.* **279**, 311 (1983).
8. H. Kimura, K. Okamoto, Y. Sakai, *J. Physiol. (London)* **365**, 103 (1985).
9. F. X. Vollenweider, M. Cuénod, K. Q. Do, *J. Neurochem.* **54**, 1533 (1990).
10. M. L. Mayer and G. L. Westbrook, *J. Physiol. (London)* **394**, 501 (1987).
11. J. R. P. Geiger *et al.*, *Neuron* **15**, 193 (1995).
12. M. Iino, S. Ozawa, K. Tsuzuki, *J. Physiol. (London)* **424**, 151 (1990).
13. L. Egebjerg and S. F. Heinemann, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 755 (1993).
14. T. A. Gilbertson, R. Scobey, M. Wilson, *Science* **251**, 1613 (1991).
15. M. Szatkowski, B. Barbour, D. Attwell, *Nature* **348**, 443 (1990).
16. J. C. Watkins and R. H. Evans, *Annu. Rev. Pharmacol. Toxicol.* **21**, 165 (1981).
17. So far, four types of EAA transporters have been identified: EAAT1(GLAST), EAAT2(GLT-1), EAAT3(EAAC1), and EAAT4 [Y. Kanai, C. P. Smith, M. A. Hediger, *Trends Neurosci.* **16**, 365 (1993); W. A. Fairman, R. J. Vandenberg, J. L. Arriza, M. P. Kavanaugh, S. G. Amara, *Nature* **375**, 599 (1995)]. The EAA-induced currents observed in voltage-clamped oocytes expressing EAAT1, -2, and -3 exhibit strong inward rectification and do not reverse below +40 mV. In contrast, the current-voltage relation of the Asp response in Purkinje cells was linear (Fig. 1D). EAAT4 is coupled with the Cl^- channel, and thus the reversal potential is reduced at higher Cl^- concentrations. In contrast, the reversal potential of the Asp response increased at higher Cl^- concentrations (Fig. 3). Furthermore, the known EAA transporters do not transport Ca^{2+}, whereas the Asp response includes a robust Ca^{2+} influx (Fig. 3).
18. Coapplication of glycine (1 μM) increased the currents induced by Asp (300 μM) to 148 ± 13% of controls in wild-type Purkinje cells (n = 4). The currents induced by Asp (300 μM) + glycine (1 μM) were reduced by a noncompetitive NMDA receptor antagonist (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801) (5 μM) and by a noncompetitive non-NMDA receptor antagonist [1-(4-aminophenyl)-4-methyl-7,8-methyl-enedioxy-5H-2,3-benzodizepine HCl] (GYKI52466) (10 μM) to 14 ± 4% and 2.3 ± 1% of controls, respectively (n = 6).
19. M. Ito, *Annu. Rev. Neurosci.* **12**, 85 (1989).
20. J. R. Brorson, P. A. Manzolillo, R. J. Miller, *J. Neurosci.* **14**, 187 (1994).
21. L. Wiklund, G. Toggenburger, M. Cuénod, *Science* **216**, 78 (1982); N. S. Nadi, D. Kanter, W. J. McBride, M. H. Aprison, *J. Neurochem.* **28**, 661 (1977).
22. D. J. Perkel, S. Hestrin, P. Sah, R. A. Nicoll, *Proc. R. Soc. London Ser. B* **241**, 116 (1990); A. Aijima, T. Hensh, R. T. Kado, M. Ito, *Neurosci. Res.* **12**, 281 (1991).
23. NR1 genotypes were determined with DNA samples from tail clips and analyzed by polymerase chain reaction (*6*).
24. Saline composition in the electrodes was 130 mM $CsMeSO_3$, 10 mM CsCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5.5 mM EGTA, 10 mM Hepes, 2 mM adenosine 5′-triphosphate (sodium salt) (adjusted to pH 7.3 with CsOH). For unambiguous identification of Purkinje cells in immature cultures, we routinely performed calbindinD28k staining (specific for Purkinje cells) (*6*). The bath solution contained 150 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 10 mM Hepes, and 20 mM glucose (pH 7.3). Mg^{2+} was omitted from the external solution throughout the experiments. Tetrodotoxin (1 μM) and picrotoxin (100 μM) were included in the solution to block spontaneous electrical activity and glycine–γ-aminobutyric acid (GABA) channels. All drugs were dissolved in the recording solution. Drugs were applied by the Y-tube method [K. Murase *et al.*, *Brain Res.* **525**, 84 (1990)] controlled by a computer (*6*). A time constant of solution exchange surrounding a neuron was estimated to be about 15 to 20 ms, judging from a rise time of Glu-induced currents. Membrane potential was corrected for the liquid-junction potential.
25. Because small currents were induced by CNQX by itself, the current trace induced by CNQX alone was digitally subtracted from that induced by Asp + CNQX from the same neuron. Although CNQX generated nonspecific currents, the blockade by CNQX of the Asp response was considered as specific antagonism of non-NMDA receptors because Asp-induced currents in Purkinje cells were also blocked by another non-NMDA receptor antagonist, 6-nitro-7-sulphamoylbenzo[f] quinoxaline-2,3-dione (NBQX) (5 μM), which did not induce current by itself (n = 3).
26. Na^+ concentration in the bath solution was reduced to 80 mM, and sucrose was used to adjust the osmolarity. Cells were exposed to the test solutions containing either 2, 5, 10, or 20 mM Ca^{2+} for 4 s at a series of membrane potentials close to the reversal potentials. Because complete solution exchange surrounding the neurons takes 15 to 20 ms (*24*), the reversal potentials measured by this method could underestimate the real value. Thus, for the determination of the reversal potentials, the current at 1 s after application of the test solution was measured.
27. In some experiments, monovalent cations in external solutions were substituted with the membrane-impermeant cation *N*-methylglucamine, and Ca^{2+} concentrations were varied (*11–13*). At −60 mV, Asp induced inward currents carried by Ca^{2+} ions at all Ca^{2+} concentrations, whereas AMPA failed to induce inward currents even at solutions containing 20 mM Ca^{2+}, confirming the specific Ca^{2+} permeability of the putative aspartate channel in NR1$^{-/-}$ Purkinje cells (n = 3). This finding further ruled out the involvement of conventional transporters (*17*) that are dependent on external Na^+ ions.
28. K. S. Pitzer and G. Mayorga, *J. Phys. Chem.* **77**, 2300 (1973).

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