mined by the cloning and subsequent sequencing of the PCR products.
7. W.-H. Li, in *Evolution of Duplicate Genes and Pseudogenes*, M. Nei and R. N. Koehn, Eds. (Sinauer, Sunderland, MA, 1983), pp. 14–37.
8. C. H. Langley, in *Population Biology of Genes and Molecules*, N. T. Takahata and J. F. Crow, Eds. (Baifukan, Tokyo, 1990), pp. 75–91; M. Kreitman, in *Evolution at the Molecular Level*, R. K. Selander, A. G. Clark, T. S. Whittam, Eds. (Sinauer, Sunderland, MA, 1991), pp. 204–221.
9. Only two length polymorphisms are observed in the 3′ flanking region of *D. yakuba*.
10. J. C. Alwine *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5350 (1977).
11. M. Long and C. H. Langley, unpublished data.
12. E. S. Kawasaki, in *PCR Protocols*, M. A. Innis *et al.*, Eds. (Academic Press, New York, 1990), pp. 21–27.
13. Total RNA was extracted from male adult flies (*D. teissieri*) and L1 larvae (*D. yakuba*) as described [D. A. Goldberg *et al.*, *Cell* **34**, 59 (1982)]. We treated the RNA with ribonuclease-free deoxyribonuclease to avoid potential DNA contamination during the PCR experiment. For RT-PCR, the *jgw*-transcribed RNA sequences were amplified from cDNA as described by Kawasaki (*12*) with the *jgw*-specific primers. The primers included the pair 5′-GGCACTCAATCCAAAGGTGTG-3′ (P1) and 5′-GCCCAAGTCCAGTTTCCAGAGT-3′ (P2) for *D. teissieri* and the pair 5′-TCCTTGAGCAACAAGAACGTAA-3′ (P3) and 5′-GTAGTTGACGGCGATGGTTGC-3′ (P4) for *D. yakuba*. The PCR products were cloned into Bluescript SK+ and sequenced by the dideoxyribonucleotide chain-terminating method.
14. *Drosophila teissieri* 5′ and 3′ ends of RNA were amplified from cDNA with the RACE technique (*27*). For the 5′ end of *jgw* RNA from *D. teissieri*, the primer used for primer extension was primer 5 (P5, 5′-GGTGGTCTCGGCAATGG-3′), which anneals to the RNA from both *jgw* and *Adh*. The cDNA generated afterwards was tailed with deoxyadenosine triphosphate. Three primers were used for the subsequent PCR reactions. They included two artificial primers (*27*) [dT17-adapter and adapter primers that are able to anneal to the artificially added poly(A) tail of cDNA] and *jgw*-specific primer 6 (P6, 5′-TCACATCGTAGGGGTAGAAGGTGACGCA-3′). For the 3′ end of *jgw* RNA in *D. teissieri*, the *jgw*-specific primer 7 (P7, 5′-CCAATTTGATTATAATGGCGCTTCG-3′) or P1 in conjunction with the dT17-adapter primer was used to amplify the 3′ portion of *jgw* RNA of *D. teissieri*. The 5′ end of *D. yakuba jgw* RNA was amplified from cDNA with P7 and *D. yakuba jgw*-specific primer P4. Genomic DNA was amplified with the use of P7 and a downstream primer; P6 was used for *D. teissieri* and primer 8 (P8, 5′-TCCAGACCAATGCCTCCCAGACCGGCAACGAAAATT-3′) for *D. yakuba*. The positions of the introns are determined by alignment of genomic and RNA sequences. Sequencing methods of the PCR products included the direct sequencing and the cloning-sequencing approaches as described (*6*).
15. J. Chelly, J.-C. Kaplan, P. Maire, S. Gautron, A. Kahn, *Nature* **333**, 858 (1988); J. Singer-Sam *et al.*, *Nucleic Acids Res.* **18**, 1255 (1990).
16. W.-H. Li, T. Gojobori, M. Nei, *Nature* **292**, 237 (1981).
17. D. T. Sullivan *et al.*, *Evol. Biol.* **24**, 107 (1990).
18. D. Lachaise *et al.*, *ibid.* **22**, 159 (1988).
19. J. H. McDonald and M. Kreitman, *Nature* **351**, 652 (1991).
20. M. Kimura, *The Neutral Theory of Molecular Evolution* (Cambridge Univ. Press, Cambridge, 1983); G. A. Watterson, *Theor. Popul. Biol.* **7**, 256 (1975); M. Nei and F. Tajima, *Genetics* **97**, 145 (1981).
21. R. R. Hudson *et al.*, *Genetics* **116**, 153 (1987).
22. E. N. Moriyama and T. Gojibori, *ibid.* **130**, 855 (1992); P. Sharp and W.-H. Li, *J. Mol. Evol.* **28**, 398 (1989).
23. M. B. Soares *et al.*, *Mol. Cell. Biol.* **5**, 2090 (1985); A. M. Weiner *et al.*, *Annu. Rev. Biochem.* **55**, 631 (1986); H.-H. M. Dahl *et al.*, *Genomics* **8**, 225 (1990); A. Ashworth *et al.*, *EMBO J.* **9**, 1529 (1990); J. Brosius, *Science* **251**, 753 (1991).
24. G. R. Fink, *Cell* **49**, 5 (1987).
25. R. L. Dorit, L. Schoenbach, W. Gilbert, *Science* **250**, 1377 (1990); L. Banyai *et al.*, *FEBS Lett.* **163**, 37 (1983).
26. We named this non-*Adh* parent gene of *jgw* as *Yande* (*ynd*) in consistency with the Chinese legend about *jingwei* (*3, 11*).
27. M. A. Frohman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8998 (1988).
28. M. Nei, *Molecular Evolutionary Genetics* (Columbia Univ. Press, New York, 1987), pp. 276–279; T. H. Jukes and C. R. Cantor, in *Mammalian Protein Metabolism*, H. N. Munro, Ed. (Academic Press, New York, 1969), pp. 21–132.
29. W.-H. Li and D. Graur, *Fundamentals of Molecular Evolution* (Sinauer, Sunderland, MA, 1991); R. R. Hudson, in *Oxford Surveys in Evolutionary Biology*, D. Futuyma and J. Antonovics, Eds. (Oxford Univ. Press, New York, 1990), vol. 7, pp. 1–40.
30. We thank J. Gillespie, A. Clark, M. Turelli, K. Burtis, and all members of our laboratory for helpful discussions and critical reading of the manuscript. D. Lachaise kindly provided the *D. teissieri* isofemale lines. We also thank P. Jeffs and M. Ashburner for sharing DNA sequences of the *Adh*-derived portions of *jgw* in *D. yakuba* and *D. teissieri* before they published these sequences (*5*). Supported by NSF (C.H.L.) and the Center for Population Biology of the University of California at Davis, a University of California at Davis Graduate Research Award, and a Jastro-Shields Research Scholarship (M.L.).

26 October 1992; accepted 4 February 1993

Modulation of Neuronal Migration by NMDA Receptors

Hitoshi Komuro and Pasko Rakic

The *N*-methyl-D-aspartate (NMDA) subtype of the glutamate receptor is essential for neuronal differentiation and establishment or elimination of synapses in a developing brain. The activity of the NMDA receptor has now been shown to also regulate the migration of granule cells in slice preparations of the developing mouse cerebellum. First, blockade of NMDA receptors by specific antagonists resulted in the curtailment of cell migration. Second, enhancement of NMDA receptor activity by the removal of magnesium or by the application of glycine increased the rate of cell movement. Third, increase of endogenous extracellular glutamate by inhibition of its uptake accelerated the rate of cell migration. These results suggest that NMDA receptors may play an early role in the regulation of calcium-dependent cell migration before neurons reach their targets and form synaptic contacts.

In the developing brain, most immature neurons migrate to their distant final destinations by extending their leading processes and translocating their soma through a terrain that is densely packed with previously generated neurons and their processes (*1*). This movement of immature neurons is essential for the establishment of normal cytoarchitecture, synaptic connectivity, and function in the brain (*2*). In the cerebellum, granule cells migrate from the site of their origin in the germinal external granular layer toward the internal granular layer along the elongated processes of Bergmann glial cells (*3*). Recently Komuro and Rakic have demonstrated that the rate of granule cell movement across the molecular layer in the cerebellum depends both on extracellular Ca^{2+} concentrations and on Ca^{2+} influx through N-type Ca^{2+} channels (*4*). However, the regulatory mechanism underlying this Ca^{2+}-dependent process remains unknown. We have now examined the role of ionotropic receptors—NMDA, non-NMDA, $GABA_A$, and $GABA_B$ (GABA is γ-aminobutyric acid)—in granule cell migration; these receptors are expressed by immature granule cells (*5*) and can directly and indirectly affect Ca^{2+} influx and intracellular Ca^{2+} concentrations (*5, 6*).

To examine whether NMDA, non-NMDA, $GABA_A$, and $GABA_B$ receptors play a significant role in the migration of granule cells, we used slice preparations of the developing mouse cerebellum stained with a lipophilic carbocyanine dye [1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)] and a laser scanning confocal microscope (*7*). Postmitotic granule cells in slice preparations migrate from the external granular layer toward the internal granular layer (Fig. 1) (*8*). Antagonists to these receptors were added to the culture medium in separate experiments. Blockade of the non-NMDA subtype of glutamate receptors [that is, kainate and AMPA (L-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors] by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (*9*), $GABA_A$ receptors by bicuculline (*6*), and $GABA_B$ receptors by phaclofen (*10*) failed to alter the rate of cell migration (Fig. 2, A and B). However, blockade of the NMDA subtype of glutamate receptor by D-2-amino-5-phosphonopentanoic acid (D-AP5) (*11*) or (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine hydrogen maleate (MK-801) (*12*) significantly decreased the

Section of Neurobiology, Yale University School of Medicine, New Haven, CT 06510.

rate of cell movement (Fig. 2, A and B).

The effects of D-AP5 and MK-801 were dose-dependent. For example, the addition of 1 to 10 μM D-AP5 to the culture medium did not significantly affect the rate of cell migration. However, higher concentrations of D-AP5 resulted in a statistically significant decrease in cell movement (Fig. 3A). In 50 μM D-AP5, the rate was 48% and in 100 μM D-AP5 it was 38% of the normal rate of cell movement (Fig. 3A).

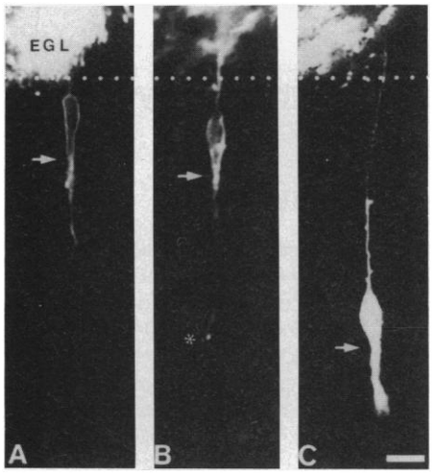

Fig. 1. Migrating granule cells in the slice preparation visualized by confocal laser microscopy. The soma of a migrating neuron as well as its leading process can be identified after exposure to DiI. The border between the external granular layer (EGL) and the molecular layer is marked by a dotted line. Cells first become bipolar (**A**), then extend the leading process (**B**), and subsequently translocate their nuclei and surrounding cytoplasm through the leading process (**C**). The range of shapes of migrating cells in the slices with lower or higher rates of movement was not different from that of the control. The asterisk shows the tip of the leading process. Scale bar, 10 μm.

We also tested the effect of changes in NMDA concentration on the rate of granule cell migration. The addition of 10 μM NMDA to the culture medium slightly enhanced the rate of cell movement, whereas the addition of 30 or 100 μM NMDA had an opposite effect (Fig. 3B). The interpretation of these latter results is difficult because the continuous application of NMDA is highly toxic (*13*) and may induce desensitization of NMDA receptors (*14*).

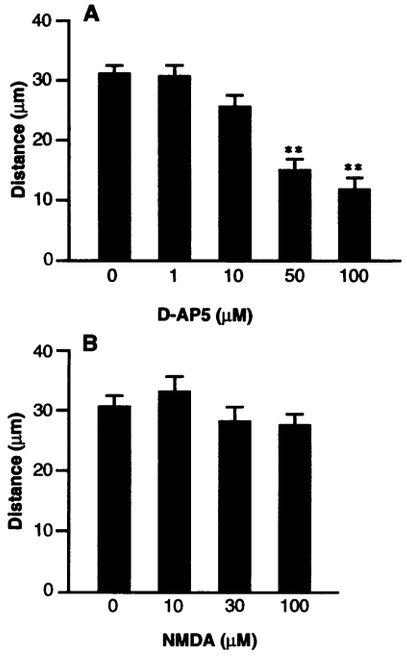

Fig. 3. The dose-dependent effect of NMDA receptor antagonist D-AP5 (**A**) and NMDA (**B**) in cell migration. Each column shows the mean length of the migration route for at least 100 labeled cells. Small bar is SEM. The slice preparations were maintained for 4 hours at 37°C after application of various concentrations of D-AP5 and NMDA. Double asterisks indicate statistical significance ($P < 0.01$).

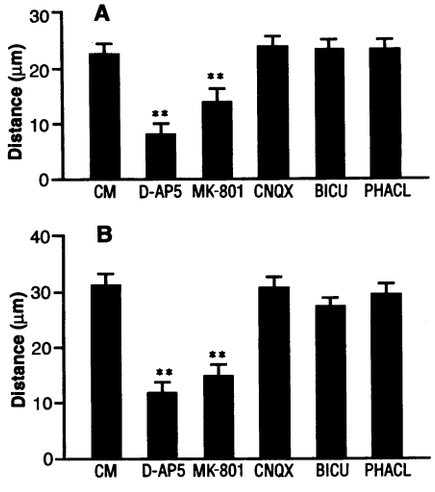

Fig. 2. The effect of antagonists to ionotropic receptors on the migration of cerebellar granule cells. All preparations were obtained from 10-day-old mice. Each column shows the mean length of the migration route for at least 100 labeled cells. Small bar is SEM. Each antagonist to specific NMDA, non-NMDA, $GABA_A$, or $GABA_B$ receptors was added to the tissue culture medium in separate experiments 2 hours after staining, and preparations were maintained for an additional 2 hours (**A**) to 4 hours (**B**). The mean distance of cell displacement after the addition of 10 μM CNQX, 10 μM bicuculline (BICU), or 500 μM phaclofen (PHACL) was not significantly different from values obtained in control slice preparations (CM) at each time point. However, addition of 100 μM D-AP5 or 10 μM MK-801 inhibited cell movement. In this and following figures, we obtained each mean migratory distance by subtracting the mean displacement of the cell soma at 2 hours in culture from the total length of the migratory pathway (*8*). Double asterisks indicate statistical significance ($P < 0.01$).

The possible involvement of the NMDA receptor in neuronal migration was further supported by the effect of Mg^{2+} and glycine on the rate of cell movement. Because extracellular Mg^{2+} blocks NMDA receptor activity in a voltage-dependent manner (*15*) and application of glycine potentiates the activity of NMDA receptors (*16*), it is expected that they both would influence cell migration. Indeed, in slice preparations maintained in Mg^{2+}-free medium, the rate of cell migration was significantly increased compared to the rate of migration of neurons in the control medium containing 0.8 mM Mg^{2+} (Fig. 4A). In contrast, the rate of movement was reduced in a high Mg^{2+} medium (10.8 mM) or in low Ca^{2+} concentrations (Fig. 4A). On the other hand, the application of 10 μM glycine significantly increased the rate of cell migration in medium with normal Ca^{2+} concentrations (Fig. 4B). Thus, granule cell mobility was highly sensitive to small fluctuations in Mg^{2+} and glycine.

Our working hypothesis is that Ca^{2+} influx through NMDA receptors and elevation of intracellular Ca^{2+} concentration in the migrating neurons are essential for their motility. Indeed, our previous (*4*) and pres-

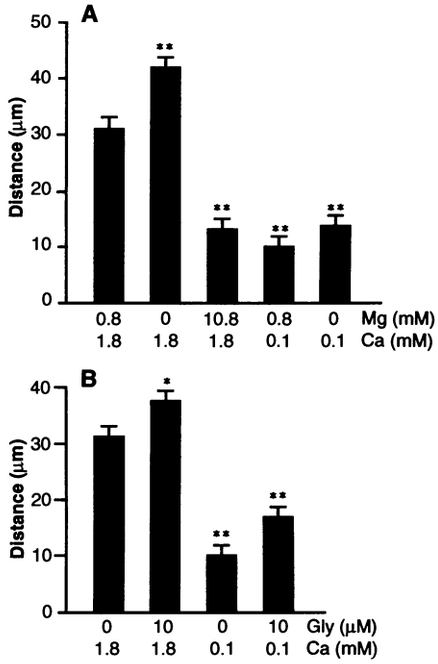

Fig. 4. The effect of low and high concentrations of Mg^{2+} (**A**) and glycine (**B**) on the rate of the granule cell migration. Each bar is an average of at least 100 cells. Small bar is SEM. The slice preparations were maintained for 4 hours after the control culture medium (0.8 mM Mg^{2+}, 0 μM glycine) was exchanged with the medium containing low or high Mg^{2+} or glycine. Single ($P < 0.05$) and double ($P < 0.01$) asterisks indicate statistical significance.

ent results on the effect of Ca^{2+}, Mg^{2+}, and glycine (Fig. 4, A and B) demonstrate that a decrease in the extracellular Ca^{2+} concentration significantly slows cell movement. To examine further the relation between the intracellular Ca^{2+} concentration and cell movement, we added to the culture medium various concentrations of the cell-permeant Ca^{2+} chelator bis-(α-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetra(acetoxymethyl)-ester (BAPTA-AM) (*17*), which clamps intracellular Ca^{2+} at low levels. At BAPTA-AM concentrations between 5 and 25 μM, there was a graded, statistically significant decrease in the rate of migration (Fig. 5A).

Because at the ages analyzed synapses on the migrating granule cells have not formed (*18*), we hypothesized that endogenous extracellular glutamate could activate NMDA receptors by nonsynaptic mechanisms. To test this possibility, we increased the extracellular glutamate concentration by adding *p*-chloromercuriphenylsulfonic acid (p-CMPS), which inhibits glutamate uptake by astrocytes (*19*). The addition of 10 to 30 μM p-CMPS to the culture medium failed to alter the rate of cell migration (Fig. 5B). However, the addition of 100 μM p-CMPS significantly increased the rate of cell movement. These results suggest that endogenous glutamate may be an important signal for the activation of NMDA receptors and that the increase of extracellular glutamate enhances the rate of cell migration until the concentration reaches the toxic level.

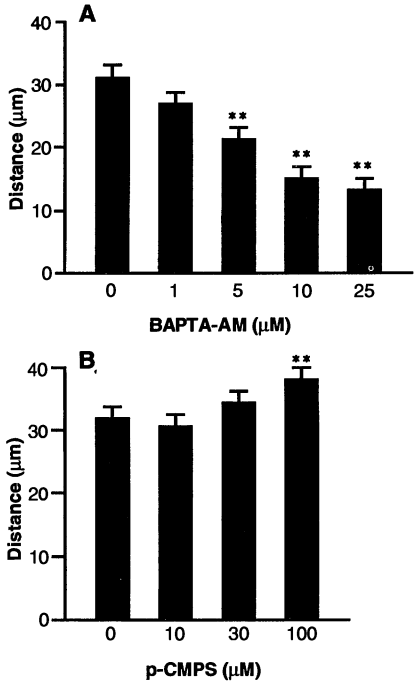

Fig. 5. The dose-dependent effect of the intracellular Ca^{2+} chelator BAPTA-AM (**A**) and the glutamate uptake inhibitor p-CMPS (**B**) on the rate of the granule cell migration. The slice preparations were maintained for 4 hours after application of various concentrations of BAPTA-AM or p-CMPS. Double asterisks indicate statistical significance ($P < 0.01$).

The role of the NMDA receptor in the regulation of neuronal migration is unexpected (this receptor has usually been associated with excitatory neurotransmission). Our results provide several lines of evidence that the rate of granule cell migration depends on the activity of NMDA receptors. Although, as far as we know, there is no information available about the emergence of this receptor on the surface of the migrating granule cells, cerebellar cells obtained largely from the external granular layer in 6-day-old rats show elevated intracellular Ca^{2+} concentrations after the addition of NMDA (*5*). Moreover, activation of the NMDA receptor in cultured granule cells affects their neurite outgrowth, morphology, and the cytoskeleton by elevating their intracellular Ca^{2+} concentration (*20*). These findings suggest that the Ca^{2+}-dependent migration of cerebellar granule cells may rely on a similar mechanism.

Parallel fibers belonging to granule cells that have already attained their final positions are a likely source of extracellular glutamate in the molecular layer of the developing cerebellum (*21*). Although granule cells eventually form synapses with Purkinje cells, migrating cells do not (*18*). Therefore, glutamate released by parallel fibers must activate the NMDA receptor of the migrating granule cells in a paracrine manner. Spontaneous, nonsynaptic activation of the NMDA receptor by extracellular glutamate has been observed in immature cortical neurons before they form synapses (*22*). The activation of this receptor, coupled to the opening of voltage-sensitive N-type Ca^{2+} channels, may initiate cell migration by influx of Ca^{2+} (*4*). Drugs that attenuate NMDA receptor activity, such as alcohol, may affect developing brain by causing defects in neuronal migration (*23*, *24*).

REFERENCES AND NOTES

1. P. Rakic, *Trends Neurosci.* **4**, 184 (1981); in *The Cell in Contact*, G. M. Edelman and J.-P. Thiery, Eds. (Wiley, New York, 1985), pp. 67–91.
2. V. S. Caviness, Jr., and P. Rakic, *Annu. Rev. Neurosci.* **1**, 297 (1978); P. Rakic, *Experientia* **46**, 882 (1990); M. E. Hatten, *Trends Neurosci.* **13**, 179 (1990).
3. P. Rakic, *J. Comp. Neurol.* **141**, 283 (1971).
4. H. Komuro and P. Rakic, *Science* **257**, 806 (1992).
5. R. D. Burgoyne, I. A. Pearce, M. Cambray-Deakin, *Neurosci. Lett.* **91**, 47 (1988); J. R. Howe, S. G. Cull-Candy, D. Colquhoun, *J. Physiol. (London)* **432**, 143 (1991); X.-Z. Zu and D.-M. Chuang, *Eur. J. Pharmacol.* **141**, 401 (1987); D. J. Laurie, W. Wisden, P. H. Seeburg, *J. Neurosci.* **12**, 4151 (1992).
6. N. Burnashev *et al.*, *Science* **256**, 1566 (1992); J. A. Connor, H.-Y. Tseng, P. E. Hockberger, *J. Neurosci.* **7**, 1384 (1987).
7. Ten-day-old mice (CD-1) were killed by rapid cervical dislocation, and their cerebella were dissected in the ice-cold Hanks' balanced salt solution (pH 7.4). Isolated cerebella were sectioned sagittally into 800-μm-thick slices with a Vibro Slice microtome (Stoelting Co.). Pia mater was carefully removed from the slice preparations. Cerebellar slice preparations were incubated for 30 min at 37°C in carbocyanine dye (DiI) (10 μg/ml) (Molecular Probes) in a cell culture medium. The incubation medium consisted of minimum essential medium (Gibco) supplemented with 40 mM glucose, 1.8 mM glutamine, 24 mM $NaHCO_3$, penicillin (90 U/ml), and streptomycin (90 μg/ml). After staining, preparations were rinsed with plain incubation medium. Slice preparations stained with DiI were maintained in the incubator (37°C, 95% air, 5% CO_2) for an additional 2 hours. Subsequently, antagonists to ionotropic receptors were added to the tissue culture medium, and the slice preparations were maintained for an additional 2 or 4 hours in an incubator. At each time point, randomly selected slice preparations were fixed with 10% formalin in 0.1 M phosphate buffer (pH 7.4) for 24 hours and sectioned at 200 μm in the sagittal plane in 0.1 M phosphate buffer (pH 7.4). Only sections obtained from the middle of the slice were used for quantitative analysis. The positions of labeled granule cells were plotted with a Bio-Rad MRC-600 confocal microscope (GHS filter block, 514-nm excitation wavelength) equipped with a Zeiss W40 objective lens (numerical aperture 0.75). Images were recorded with a Panasonic TQ-3031 optical disk recorder or a Racet cosmos 600 optical drive. We estimated the length of the migratory pathway by measuring the distance from the front of labeled cell soma (indicated by arrows in Fig. 1) to the border between the external granular and the molecular layers (dotted line in Fig. 1).
8. The mean length of the migration route for labeled granule cells after 2 hours without the addition of any antagonist to ionotropic receptors was 33.0 ± 1.2 μm (mean ± SEM) (n = 191). After 4 hours the mean length of the migrating route was 55.6 ± 1.6 μm (n = 183) and after 6 hours, 63.8 ± 1.7 μm (n = 155). These mean lengths of granule cell migration in slice preparation are comparable to the lengths measured in vivo (*4*).
9. T. Honoré *et al.*, *Science* **241**, 701 (1988).
10. H. Hasuo and J. P. Gallagher, *Neurosci. Lett.* **86**, 77 (1988).
11. H. J. Olverman, A. W. Jones, J. C. Watkins, *Nature* **307**, 460 (1984).
12. J. E. Huettner and B. P. Bean, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1307 (1988).
13. D. W. Choi, in *Glutamate, Cell Death and Memory*, P. Ascher, D. W. Choi, Y. Christen, Eds. (Springer-Verlag, Berlin, 1991), pp. 125–136.
14. I. V. Chizhmakov, N. I. Kiskin, O. A. Krishtal, *J. Physiol. (London)* **448**, 453 (1992).
15. L. Nowak, P. Bregestovski, P. Ascher, A. Herbet, A. Prochiantz, *Nature* **307**, 462 (1984).
16. J. W. Johnson and P. Ascher, *ibid.* **325**, 529 (1987).
17. M. A. Cambray-Deakin and R. D. Burgoyne, *Dev. Brain Res.* **66**, 25 (1992).
18. P. Rakic and R. L. Sidman, *J. Comp. Neurol.* **152**, 103 (1973).
19. D. R. Curtis, A. W. Duggan, G. A. R. Johnston, *Exp. Brain Res.* **10**, 447 (1970); V. J. Balcar and G. A. R. Johnston, *J. Neurochem.* **19**, 2657 (1972); A. Schousboe *et al.*, *Prog. Brain Res.* **94**, 199 (1992).
20. N. A. Rashid and M. A. Cambray-Deakin, *Dev. Brain Res.* **67**, 301 (1992); I. A. Pearce, M. A. Cambray-Deakin, R. D. Burgoyne, *FEBS Lett.* **223**, 143 (1987).
21. G. Levi, M. Patrizio, V. Gallo, *J. Neurochem.* **56**, 199 (1991).
22. M. G. Blanton and A. R. Kriegstein, *J. Neurophysiol.* **67**, 1185 (1992).
23. D. M. Lovinger, G. White, F. F. Weight, *Science* **243**, 1721 (1989).
24. S. K. Clarren, E. C. Alvord, Jr., S. M. Sumi, *J. Pediatr.* **92**, 64 (1978).

25. We thank R. Cameron, P. S. Goldman-Rakic, D. Kornack, and K. Wikler for useful suggestions and comments on the manuscript. This research was supported by the U.S. Public Health Service.

26 October 1992; accepted 14 January 1993