probed with the plasmid pUDG0603 [H. Kodama *et al.*, *Chromosoma* **96**, 18 (1987)]. This plasmid contains a member of a repetitive element that is unique to the W chromosome. The presence of the W chromosome in the sex-reversed chickens identifies them as genetic females.

27. We thank S. Mizuno for providing plasmid pUDG0603, M. J. McPhaul, and J. D. Wilson for the chicken aromatase cDNA clone, and G. M. Mark and S. Zavodny for expressing the aromatase cDNA. In addition, we thank E. Mills for help with the chickens, M. Harper for preparation of the manuscript, and D. Lamb for assistance in interpreting the histology of the gonads.

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High Probability Opening of NMDA Receptor Channels by L-Glutamate

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Synaptic plasticity can be triggered by calcium flux into neurons through synaptically activated *N*-methyl-D-aspartate (NMDA) receptor channels. The amplitude and time course of the resulting intracellular calcium transient depend on the number of open NMDA receptor channels and the kinetics of their activation. Short applications of L-glutamate to outside-out patches from hippocampal neurons in the presence and absence of MK-801 revealed that about 30 percent of L-glutamate–bound channels are open at the peak of the current. This high probability of opening suggests that very few channels are required to guarantee a large, localized postsynaptic calcium transient.

SYNAPTIC ACTIVATION OF NMDA receptor channels leads to a slowly rising, long-lasting excitatory postsynaptic current (EPSC) at a variety of excitatory synapses in the vertebrate central nervous system (*1–8*). The slow time course of the NMDA receptor EPSC is caused by a slow opening rate of NMDA channels after binding the transmitter L-glutamate and by repetitive opening resulting from slow unbinding (*7–9*). Although the kinetics of NMDA receptor channel openings in the continuous presence of agonists have been studied in detail (*10–12*), sudden jumps in agonist concentrations evoke desensitizing responses that cannot be easily predicted from steady-state recordings (*7–9, 13, 14*). These characteristics of channel gating are also difficult to address with the use of synaptic preparations because presynaptic release parameters are not readily controlled. In the experiments described here outside-out patches from hippocampal neurons were used as surrogate synapses to determine opening probability while controlling variables such as receptor saturation (*7–9, 15*). By using the anticonvulsant MK-801 (*16*), which blocks only open NMDA receptor channels [and in the time frame of these experiments, blocks them irreversibly (*17–19*)], the probability that a liganded channel will open and the open probability at the peak of the current can be estimated.

The superimposed records in Fig. 1A are averages of responses of an outside-out patch evoked by short, saturating applications of L-glutamate [1 mM for 8 ms (*7–9*)] in the presence of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (2 μM). The time course of these currents is the same as that of the NMDA receptor component of EPSCs recorded in cultured neurons (*7, 8*) and those in the hippocampal slice (*4–6*). If both the control solution and the L-glutamate solution were switched to solutions containing 20 μM MK-801, a single 8-ms application of L-glutamate resulted in a current attenuated in both amplitude and duration (Fig. 1B). Ten seconds after activating this current, the solutions were exchanged for those without MK-801 and the patch was washed with control solution for 30 s. Subsequent L-glutamate applications evoked currents that were reduced in amplitude relative to pre–MK-801 trials (Fig. 1C), but with similar time courses (Fig. 1D). Because high concentrations of MK-801 were used, most of the channels that were opened by this single pulse of L-glutamate should have been blocked by MK-801. Only the channels that were bound by agonist but never actually opened, or opened for a short time, would be available for subsequent activation. The percentage decrease in charge transfer produced by a single application of L-glutamate in the presence of MK-801 was 70 ± 3.6% (mean ± SE) in 20 μM MK-801 (n = 8), 65 ± 2.2% in 10 μM MK-801 (n = 7), and 46 ± 5.4% in 3 μM MK-801 (n = 5). The percent inhibition of charge transfer at the highest concentration provides an estimate of the probability that a channel bound by L-glutamate will open before L-glutamate unbinds ($P_{o(total)}$ = 0.7) (*20*). This estimate depends on the assumptions that MK-801 only blocks channels that are open and that the block is irreversible at negative holding potentials over this period of time (*17–19*). A plot of the charge transfer evoked by each application of L-glutamate versus time demonstrates these phenomena (Fig. 1E). When the patch was exposed to MK-801 but not in conjunction with L-glutamate, responses to L-glutamate after washing out MK-801 were not diminished (Fig. 1A). In nine trials (eight patches), applications of MK-801 alone had no effect on subsequent responses to L-glutamate (P = 0.44; paired t test). In addition, after a single L-glutamate application in the presence of MK-801, subsequent responses to L-glutamate remained stable, indicating that recovery from MK-801 blockade was not significant in these conditions (Fig. 1E).

If the probability of a bound channel

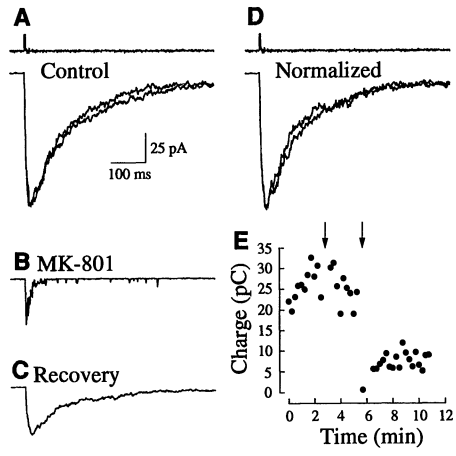

Fig. 1. L-glutamate activates NMDA receptor channels with a high probability. (**A**) Superimposed averages of currents evoked by 8-ms applications of 1 mM L-glutamate in an outside-out patch in the continued presence of 20 μM glycine. The two averages represent responses recorded before (average of 11 responses) and after exposure to 20 μM MK-801 (9 responses) in the absence of L-glutamate. (**B**) Single response of the same patch as in (A) to 1 mM L-glutamate in the continuous presence of MK-801 (20 μM). (**C**) Averaged response of the same patch to L-glutamate after superfusing the patch with control solution for 30 s. (**D**) Superimposition of averaged currents recorded before and after the single L-glutamate response in the presence of MK-801. The averaged current in (C) has been normalized to the peak amplitude of the response in (A). The similarity in time courses of the two averages suggests that channels with similar kinetic properties underlie both responses. (**E**) Charge transfer of individual responses to L-glutamate in the same patch before and after MK-801 treatment which did (right arrow) and did not (left arrow) coincide with an application of L-glutamate. Charge transfer was measured from records 1024 ms long. The top records in (A) and (D) are open-tip currents (*15*).

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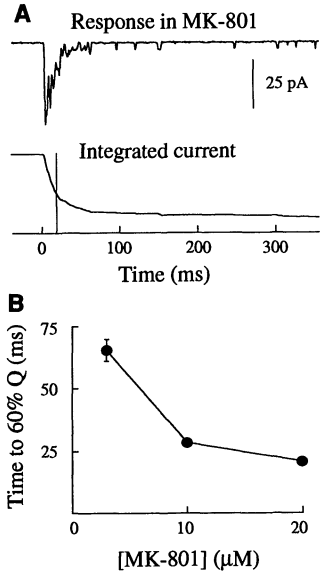

Fig. 2. First latency distribution of NMDA receptor channel openings. (**A**) The current evoked by L-glutamate in the presence of MK-801 (top; same as in Fig. 1B) is integrated in time (lower) and represents a cumulative history of the first openings of channels after L-glutamate application. The thin vertical line in the lower record marks 20.9 ms after the beginning of the L-glutamate application. (**B**) The time required for 60% of the total charge transfer to occur in the presence of 3, 10, and 20 μM MK-801. Error bars for 10 and 20 μM MK-801 are within the symbols.

opening is 0.7, what is the probability that it will be open at the peak of the response, which is reached in about 20 ms? If the NMDA receptor current evoked by L-glutamate in the continuous presence of MK-801 is integrated in time, the resulting distribution represents the time course of charge transfer (Fig. 2A). Sixty percent of the charge transfer occurred in 20.9 ± 0.9 ms after the beginning of the L-glutamate application in 20 μM MK-801 (n = 8). Decreasing the MK-801 concentration increased the period required to reach 60% charge transfer. Because the period increased only slightly from 20 to 10 μM (Fig. 2B), the time course of the charge transfer in 20 μM MK-801 should represent a cumulative first latency distribution, that is, the time course over which NMDA receptor channels opened for the first time after becoming bound by L-glutamate. Thus, of the channels that opened, the probability of opening for the first time by the time of the peak response was 0.6. Burst or cluster length of NMDA receptor channel openings is about 10 to 30 ms (*10–12*); thus, many of the channels that opened during the first 20 ms would still be open at the peak of the control current. An upper limit of the peak open probability is obtained if it is assumed that all of the channels that opened in the first 20 ms were still open at the peak of the control response. Then the peak open probability would be 0.42 (that is, the probability of a channel opening in the first 20 ms multiplied by the probability of a channel opening at any time after binding L-glutamate). An estimate of the true probability of a channel being open at the peak of the current can be calculated if the rate at which MK-801 blocks the channel can be determined.

The rate of channel block by MK-801 was determined by measuring the mean lifetime of channel events in the presence of constant concentrations of MK-801. Because the mean dwell time in any state is the reciprocal of the sum of rates for leaving that state, then, assuming a single open state, the mean open time O is described by

$$O = 1/(r[\text{MK-801}] + \alpha) \quad (1)$$

where r and α are the rate constants of MK-801 block and of normal closing of NMDA receptor channels, respectively. Mean open time was estimated from the time constants of single exponential fits of open time distributions (Fig. 3A) and r was determined from the slope of the least-squares fit of the reciprocal of mean open times at different concentrations of MK-801 (*21*) (Fig. 3B). This yielded a blocking rate of 23.7 $\mu M^{-1}\ s^{-1}$.

With this estimate of the blocking rate r, the probability of opening at the peak of a response to a short, saturating application of L-glutamate can be estimated from

$$P_{o(peak)} = (I_p P_{o(total)})/(Q_{MK} r[\text{MK-801}]) \quad (2)$$

where I_p is the peak amplitude of the response, $P_{o(total)}$ is the probability of a bound channel opening before unbinding, and Q_{MK} is the charge transfer in the presence of MK-801 (*22*). Using Eq. 2, $P_{o(peak)}$ was calculated to be 0.27 ± 0.017 in 10 μM MK-801 and 0.28 ± 0.028 in 20 μM MK-801 (n = 7 and 8, respectively). Thus, at the peak of the current evoked by L-glutamate, about 30% of all NMDA receptor channels in the patch are open (*23*). This is a surprisingly high number because Huettner and Bean (*17*) estimated the probability of opening in steady-state conditions to be at most 0.002. The difference can be accounted for by the saturating concentrations of L-glutamate and glycine used in the present study, by receptor desensitization, which is dramatic with prolonged agonist applications to outside-out patches (*8, 13, 17*), and by rundown of channel activity that is usually observed in patches exposed to millimolar extracellular calcium in the presence of agonist for prolonged periods (*24*).

The results show that, once NMDA receptor channels become bound by L-glutamate and glycine, 70% of them will open before L-glutamate unbinds. Of those channels that do open, 60% open for the first time by the time of the peak of the response, or within about 20 ms after binding L-glutamate. This indicates that the prolonged currents activated by short applications of L-glutamate are mainly due to repetitive bursts and clusters of openings of channels (*7–12, 25*) that first opened soon after L-glutamate binding.

Spontaneous transmitter release at synapses between hippocampal neurons activates few NMDA receptor channels (*3*). This suggests that if synaptic NMDA receptors behave the same as extrasynaptic receptors (*2, 7, 8, 26*), the number of channels that become bound by spontaneously released L-glutamate is small. Recent reports have suggested that evoked transmitter release may saturate postsynaptic receptors at central synapses (*27, 28*). If spontaneous release saturates postsynaptic NMDA receptors, then it follows that few NMDA receptors even exist in the postsynaptic membrane adjacent to each release site. The apparent role of NMDA receptors in the induction of long-term potentiation is to mediate a transient increase in intracellular calcium (*29, 30*) by providing a highly calcium-permeable conductance in the plasma membrane (*2, 25, 31, 32*). Once synaptic NMDA re-

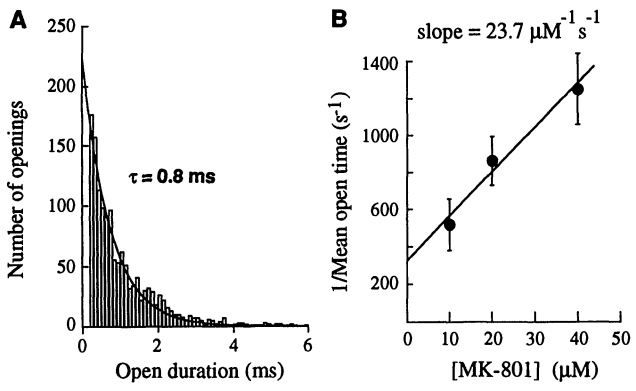

Fig. 3. Blocking rate of NMDA receptor channels with MK-801. (**A**) Open time distribution from recordings in the presence of 40 μM MK-801. The distribution was fitted with a single exponential with a time constant of 0.8 ms; 100-μs bin width. (**B**) Reciprocal of mean open time as a function of MK-801 concentration. The line is the least-squares fit by the equation $1/O = r[\text{MK-801}] + \alpha$. Each point is mean ± SEM from three patches.

ceptors are bound by transmitter, a high probability of opening would guarantee large, localized postsynaptic calcium transients and thus facilitate the activation of calcium-dependent processes such as long-term potentiation (*29, 30*).

REFERENCES AND NOTES

1. N. Dale and A. Roberts, *J. Physiol.* **363**, 35 (1985).
2. I. D. Forsythe and G. L. Westbrook, *ibid.* **396**, 515 (1988).
3. J. M. Bekkers and C. F. Stevens, *Nature* **341**, 230 (1989).
4. S. Hestrin, P. Sah, R. A. Nicoll, *Neuron* **5**, 247 (1990).
5. A. D. Randall, J. G. Schofield, G. L. Collingridge, *Neurosci. Lett.* **114**, 119 (1990).
6. B. U. Keller, A. Konnerth, Y. Yaari, *J. Physiol.* **435**, 275 (1991).
7. R. A. J. Lester, J. D. Clements, G. L. Westbrook, C. E. Jahr, *Nature* **346**, 565 (1990).
8. R. A. J. Lester and C. E. Jahr, *J. Neurosci.*, in press.
9. J. D. Clements and G. L. Westbrook, *Neuron* **7**, 605 (1991).
10. P. Ascher, P. Bregestovski, L. Nowak, *J. Physiol.* **399**, 207 (1988).
11. J. R. Howe, S. G. Cull-Candy, D. Colquhoun, *ibid.* **432**, 143 (1991).
12. A. J. Gibb and D. Colquhoun, *Proc. R. Soc. London* **243**, 34 (1991).
13. W. Sather, J. W. Johnson, G. Henderson, P. Ascher, *Neuron* **4**, 725 (1990).
14. L. Vyklicky, Jr., M. Benveniste, M. L. Mayer, *J. Physiol.* **428**, 313 (1990).
15. Experiments were performed on hippocampal neurons from 1- to 3-day-old neonatal rats maintained in primary cell culture for 1 to 3 weeks [as described (*7, 8*)]. Outside-out patch recordings (Axopatch-1C, Axon Instruments, Foster City, CA) from hippocampal neurons were obtained with the use of pipettes containing (in mM): cesium or sodium gluconate or methanesulfonate, 140; NaCl, 10; Hepes, 10; EGTA, 10; and Mg adenosine triphosphate (ATP), 4; adjusted to pH 7.2 with CsOH or NaOH. Control external solutions contained (in mM): NaCl, 160; KCl, 3; Hepes, 5; glycine, 0.02; and CNQX, 0.002; adjusted to pH 7.4 with NaOH. External Ca^{2+} was 0.2 mM to help prevent slow rundown of NMDA receptor channel currents. External solutions were gravity fed into each of the four lumens of four-barreled glass tubing (Vitro Dynamics, Rockaway, NJ), which was pulled to an overall tip diameter of about 200 μm. The patch was positioned within 50 μm of the tip, near the interface formed between the continuously flowing control and drug solutions. The solution exchange was made by rapidly moving the solution interface across the tip of the patch pipette using a piezoelectric translator (Physik Instrumente, Waldbronn, Germany, model P245.30) attached to the flow tube. Outside-out patches were clamped at −60 mV, and the currents were low pass-filtered at 0.5 to 1.0 kHz and digitally sampled at 1 to 2 kHz. Open time histograms were from recordings filtered at 2 kHz and sampled at 20 kHz. High purity salts were obtained from Aldrich Chemical (gold label). Other chemicals were obtained from Sigma except for CNQX (Cambridge Research Biochemicals and Tocris), glycine (Bio-Rad, Richmond, CA), and MK-801 (Merck Sharp and Dohme Research Center). The speed and completeness of solution changes were tested at the end of each recording by "blowing out" the membrane and monitoring the open tip current caused by differences in liquid junction potentials in the control and drug solutions (the drug solution was diluted with water 50:1). Experiments were performed at room temperature (25°C).
16. E. H. F. Wong *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7104 (1986).
17. J. E. Huettner and B. P. Bean, *ibid.* **85**, 1307 (1988).
18. R. F. Halliwell, J. A. Peters, J. J. Lambert, *Br. J. Pharmacol.* **96**, 480 (1989).
19. J. F. MacDonald *et al.*, *J. Physiol.* **432**, 483 (1991).
20. The probability that a bound channel opens before it becomes unbound ($P_{o(total)}$) was estimated from $P_{o(total)} = 1 -$ (charge transfer after MK-801)/(charge transfer before MK-801).
21. At 10 μM MK-801, the open time histogram was described better by a double rather than a single exponential. The mean open time used in Fig. 3B for 10 μM was therefore the arithmetic mean of the measured open durations (half-height criteria), which is a slight overestimate of the true mean open time; thus, the slope of the fit (rate constant r) is slightly steeper than it should be ($\alpha = 324$).
22. The probability of channel opening at the peak of the current is $P_{o(peak)} = N_p/N_t$, where N_p is the number of channels open at the peak and N_t is the total number of channels in the patch. $N_p = I_p/i$, where I_p is the amplitude of the peak current and i is the single-channel current amplitude. Because the number of channels that are opened, on average, by a single application of L-glutamate is $N_o = N_t P_{o(total)}$, then $N_t = N_o/P_{o(total)}$. The mean charge transfer evoked by applications of L-glutamate before MK-801 is given by $Q_{(control)} = iTON_o$, where TO is the mean total amount of time that channels that do open spend in the open state before they become unliganded, and thus $N_o = Q_{(control)}/(iTO)$. TO can be estimated as $TO = Q_{(control)}/(Q_{MK}r[MK\text{-}801])$, where Q_{MK} is the charge transfer in the presence of MK-801. Thus, $P_{o(peak)} = (I_p P_{o(total)})/(Q_{MK}r[MK\text{-}801])$. TO was calculated to be 91.1 ± 8.7 ($n = 15$).
23. Using a model that can approximately account for NMDA receptor channel activation and desensitization by a number of agonists (*8*), we fitted control responses to L-glutamate and those in MK-801 by assuming a $P_{o(peak)}$ of 0.3 and a blocking rate of 22 $\mu M^{-1}\ s^{-1}$.
24. C. Rosenmund, P. Legendre, G. L. Westbrook, *Soc. Neurosci. Abstr.* **17**, 957 (1991); C. E. Jahr, unpublished observations.
25. C. E. Jahr and C. F. Stevens, *Nature* **325**, 522 (1987).
26. H. P. C. Robinson, Y. Sahara, N. Kawai, *Biophys. J.* **59**, 295 (1991).
27. F. A. Edwards, A. Konnerth, B. Sakmann, *J. Physiol.* **430**, 213 (1990).
28. A. Larkman, K. Stratford, J. Jack, *Nature* **350**, 344 (1991).
29. G. Lynch, J. Larson, S. Kelso, G. Barrionuevo, F. Schottler, *ibid.* **305**, 719 (1983).
30. R. C. Malenka, J. A. Kauer, R. S. Zucker, R. A. Nicoll, *Science* **242**, 81 (1988).
31. A. B. MacDermott, M. L. Mayer, G. L. Westbrook, S. J. Smith, J. L. Barker, *Nature* **321**, 519 (1986).
32. P. Ascher and L. Nowak, *J. Physiol.* **399**, 247 (1988).

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Axon Guidance by Gradients of a Target-Derived Component

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Spatial gradients of axon guiding molecules have long been suspected to provide positional and directional cues for retinal ganglion cell axons growing within the optic tectum. With the identification of a guiding activity from tectal cell membranes, it has become possible to investigate the potential physiological significance of molecular gradients for retinal growth cone behavior in vitro. A subset of retinal growth cones, those from the temporal half, were highly sensitive to small concentration changes of the guiding component. The degree of response was correlated with the strength of the gradient. These findings demonstrate that the neural growth cone can read gradients of surface-associated information.

DURING NERVOUS SYSTEM DEVELopment elongating axons can be guided to their respective targets by chemical cues (*1*). Concentration gradients of specific molecules have been recognized as a plausible means to generate directional information for axonal pathfinding. Chemotropic factors, for example, could be secreted by the target region to form a diffusion gradient within which the motile tips of the axons, the growth cones, may orient (*2*). Alternatively, local positional cues could be differentially distributed along axonal pathways (*3*). The retinotectal map could also be generated by a similar mechanism (*4*). Retinal ganglion cells project their axons to the optic tectum so that their terminations form a reversed image of the retina. The temporal half of the retina projects to the anterior part of the tectum, the nasal retina to the posterior tectum. Precise mapping also occurs along the dorsoventral axes.

Although it is now widely accepted that retinotectal specificity is based on "chemoaffinity" (*5*) between retinal growth cones and their target cells, the cellular mechanisms governing this process are unknown. Anatomical data suggest that there is a long-range guidance of growth cones within the tectum that could well be achieved by chemical gradients (*6*). Theoretically, a small set of graded cues in the tectum could specify both direction and target position, provided that the retinal growth cones carry differential sets of corresponding receptors (*7*). Growth cone steering may depend on the functional equivalents of attractive (pulling)

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