20. S. G. Miller and M. B. Kennedy, *Cell* **44**, 861 (1986); T. Saitoh and J. H. Schwartz, *J. Cell Biol.* **100**, 835 (1985).
21. R. E. Akers, D. M. Lovinger, P. A. Colley, D. J. Linden, A. Routtenberg, *Science* **231**, 587 (1986); G.-Y. Hu *et al.*, *Nature* **328**, 426 (1987); D. M. Lovinger, P. A. Colley, R. F. Akers, R. B. Nelson, A. Routtenberg, *Brain Res.* **399**, 205 (1986); D. V. Madison, R. Malinow, R. W. Tsien, *J. Physiol. (London)* **398**, 18P (1988); R. C. Malenka, D. V. Madison, R. A. Nicoll, *Nature* **321**, 175 (1986).
22. G. Lynch and M. Baudry, *Science* **224**, 1057 (1984).
23. We thank D. Perkel and P. Sah for their comments on the manuscript and D. Perkel for his help in setting up the computer routines for storage and analysis of the data. Supported by NIH grants MH38256, MH00437, and NS24205.

13 June 1988; accepted 2 August 1988

Muscarinic Depression of Long-Term Potentiation in CA3 Hippocampal Neurons

STEPHEN WILLIAMS AND DANIEL JOHNSTON

Behavioral studies have suggested that muscarinic cholinergic systems have an important role in learning and memory. A muscarinic cholinergic agonist is now shown to affect synaptic plasticity in the CA3 region of the hippocampal slice. Long-term potentiation (LTP) of the mossy fiber–CA3 synapse was blocked by muscarine. Low concentrations of muscarine (1 micromolar) had little effect on low-frequency (0.2 hertz) synaptic stimulation but did significantly reduce the magnitude and probability of induction of LTP. Experiments under voltage clamp showed that muscarine blocked the increase in excitatory synaptic conductance normally associated with LTP at this synapse. These results suggest a possible role for cholinergic systems in synaptic plasticity.

LONG-TERM POTENTIATION (LTP) IS a form of use-dependent synaptic plasticity that is an attractive candidate as a substrate for learning and memory (*1*). LTP is a prominent feature of all excitatory synapses studied so far in the hippocampus, an area of critical importance for certain types of memory processing (*2*). Lesion of the cholinergic afferents to the hippocampus, the fimbria of the fornix (*3*), results in learning deficits in animals, an impairment that can be mimicked by cholinergic antagonists (*4*). Furthermore, memory deficits occurring with certain senile dementias have an associated diminishment of cholinergic function (*5*). We have investigated the effects of the cholinergic agonist muscarine on LTP in the voltage-clamped mossy fiber–CA3 pyramidal cell synapse of the hippocampus, a synapse particularly suitable for detailed biophysical analysis (*6*).

Transverse hippocampal slices (450 μm) were prepared from adult rats and maintained in vitro, at 32° to 34°C, with standard techniques (*6–9*). Conventional single-electrode voltage-clamp experiments were performed with low-resistance microelectrodes (20 to 40 megohms). Picrotoxin (10 μ*M*) was added routinely to the bathing medium to block inhibitory postsynaptic currents, thereby allowing unambiguous measurement of the excitatory postsynaptic potentials (EPSPs) and currents (EPSCs) (*6, 8*); to prevent epileptiform discharges, 3 m*M* Ca^{2+} and 3 m*M* Mg^{2+} were present in the bathing medium (*8, 9*). Measurements of EPSPs were made between −70 and −90 mV (constant for any given cell) to prevent action potential generation. At this hyperpolarized holding potential, muscarine had little effect on input resistance, in contrast to potentials close to and positive to the resting potential (*10*), thereby minimizing consequent changes in EPSP amplitude. Data are based on 105 stable impalements (mean input resistance, 52 ± 2 megohms), 31 of which were maintained for at least 20 min after tetanic stimulation. LTP was defined as >20% increase in the synaptic response maintained for at least 15 min after (post) tetanic stimulation (15′ PT) (*7*). Comparisons between data sets were made with an appropriate *t* test, and data both in the text and figures are presented as mean ± SEM. Significance level was set at $P < 0.05$.

The CA3 pyramidal neuron of the hippocampus receives three major excitatory synaptic inputs: the mossy fiber, commissural, and recurrent pathways. It has been shown by extracellular measurement that LTP of the putative mossy fiber synaptic response is not blocked by 2-amino-5-phosphonovalerate (APV) (*11*), an *N*-methyl-D-aspartate (NMDA) antagonist, but LTP of the commissural-associational input to CA3 is blocked by APV (*11, 12*). As a test of our criteria for identifying mossy fiber responses (*6, 8*) (which were the location of stimulating electrode and EPSC kinetics), several experiments were performed in the presence of APV. We found that APV (10 μ*M*, $n = 5$; 50 μ*M*, $n = 2$) had no significant effect on the control EPSP or EPSC amplitude, and LTP could still be evoked (mean EPSP increase +71 ± 17%, $n = 4$). There was no significant difference between LTP obtained in control cells compared to that in cells tetanized in the presence of APV, confirming that the observed LTP is not dependent on NMDA receptor activation.

In control cells, synaptic responses were constant over time (up to 30 min); after

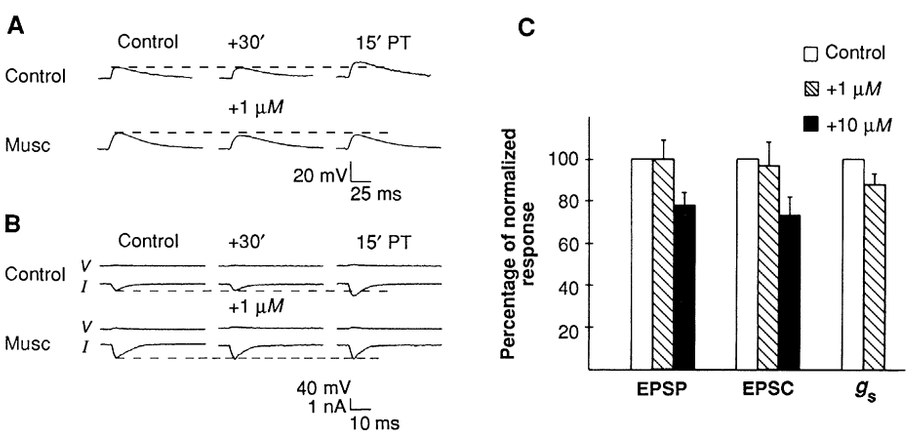

Fig. 1. The effects of muscarine on synaptic transmission and LTP. (**A**) Upper traces show that in a control cell the EPSP is stable for 30 min (30′), but that 15 min after tetanus (15′ PT) an increase in amplitude is seen. The lower traces (musc, different cell) show that 1 μ*M* muscarine produced a small depression of the EPSP before tetanus and prevented the development of LTP at 15′ PT. (**B**) In a similar experiment under voltage clamp (*V*, membrane potential; *I*, clamp current), LTP in the control cell was seen as an increase in the EPSC, but in the muscarine-treated cell no increase was apparent. (**C**) Summary of the actions of muscarine (1 and 10 μ*M*) on synaptic transmission in the absence of tetanus. All data were normalized to control. EPSP: 1 μ*M*, $n = 10$; 10 μ*M*, $n = 6$. EPSC: 1 μ*M*, $n = 8$; 10 μ*M*, $n = 5$. g_s: 1 μ*M*, $n = 5$.

Program in Neuroscience, Section of Neurophysiology, Department of Neurology and Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, TX 77030.

tetanus, however, there was a marked, sustained increase in EPSP amplitude (Fig. 1A). LTP was seen in 13 of 15 control cells (mean EPSP increase +50 ± 9%, n = 15). In contrast, only 3 of 10 cells tetanized in the presence of 1 μM muscarine showed LTP (mean EPSP increase +7 ± 9%, n = 10). Under voltage clamp, 11 of 12 control cells showed LTP (mean EPSC increase +43 ± 11%) compared to 1 of 7 muscarine-treated cells (mean EPSC increase +6 ± 6%). In the cell shown in Fig. 1A, the addition of muscarine (1 μM) produced a small depression of the EPSP and prevented the development of LTP. (In control and muscarine-treated cells, the stimulus amplitude for the tetanus train was adjusted such that it evoked an EPSP of 10 mV for a single stimulus to compensate for any changes in EPSP amplitude induced by muscarine before the tetanus.) In another cell, under voltage clamp, there was little effect of 1 μM muscarine on the EPSC (Fig. 1B), but again LTP was not obtained. At a higher dose (10 μM), muscarine produced a significant depression of synaptic transmission (Fig. 1C), as previously reported in the CA1 region (*13*). At 1 μM this effect was sometimes apparent but much less pronounced (Fig. 1C), and in a few cells 1 μM actually increased the EPSP. In the absence of tetanus the effects of muscarine were reversed after 20 to 45 min of wash. In four experiments 1 μM muscarine was added immediately after the tetanus and continuously perfused for up to 30 min. In these experiments LTP was observed (mean EPSP increase +42 ± 7%).

In Fig. 2 the time course of the EPSP amplitudes is shown before and after the tetanus. The EPSP amplitudes are plotted versus time, pooling data from all the control cells (Fig. 2A). The EPSP amplitudes are fairly stable with time, but after tetanus, given at the 0 time point, there is a marked potentiation, shown in Fig. 2A when measured at 5, 10, 15, and 30 min after tetanus. All time points after tetanus were significantly larger than control (paired t test, with Bonferroni correction). With the application of 1 μM muscarine (Fig. 2B, pooled data from all muscarine-treated cells), no potentiation was observed after 15 min. Some potentiation may have occurred after tetanus at the 5-min time point, but this was not significantly larger than control.

In most experiments, input-output (I-O) curves were constructed for a range of stimulus intensities before and after tetanus. In a control cell (Fig. 3A) after tetanus there was a pronounced leftward shift in the I-O relation, indicating the development of LTP. This shift was also observed under voltage clamp (Fig. 3B, different cell). Muscarine (1 μM) produced in some cells a slight leftward shift in the I-O curve of the EPSP before tetanus (Fig. 3C) but still prevented the development of LTP. A similar block of LTP was observed under voltage clamp in another cell in which muscarine had little effect on the EPSC amplitude before tetanus (Fig. 3D).

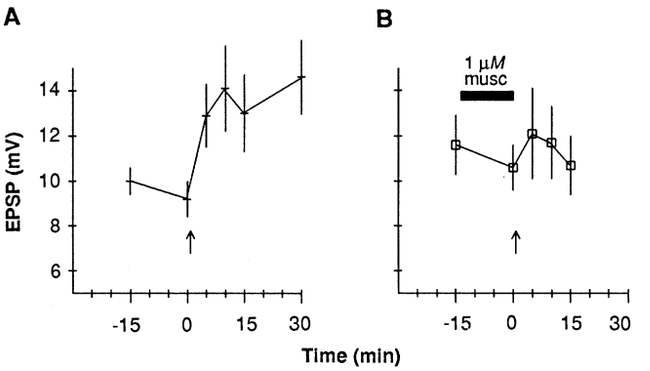

Fig. 2. Time course of the change in the amplitudes of the synaptic responses before and after tetanus. In each experiment a stimulus intensity was chosen that evoked an approximately 10-mV EPSP to a test pulse. The time course of changes in the response to this test stimulus is plotted. (**A**) Data are pooled from ten control cells and each point is a mean ± SEM. The first two time points are before tetanus controls, 15 min and just prior to tetanus. All responses after tetanus were significantly larger than before tetanus control values (paired t test, with Bonferroni correction). (**B**) Data pooled from ten muscarine-treated cells. The first time point shows control EPSP and the second is the before tetanus EPSP in the presence of 1 μM muscarine. After tetanus no significant changes were observed in EPSP amplitude. (Stimulus trains are indicated by the arrows.)

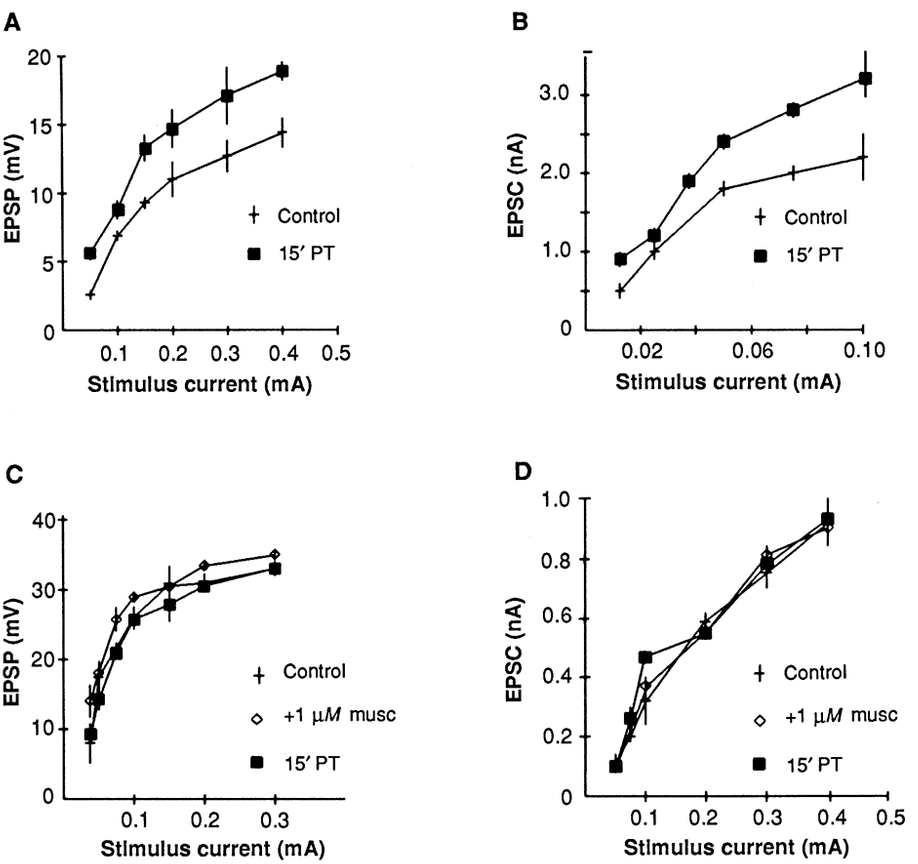

Fig. 3. Synaptic I-O relations measured under current and voltage clamp. (**A**) Control cell exhibiting LTP under current clamp. Peak EPSP amplitude is plotted versus stimulus current under control conditions and 15 min after tetanus. (**B**) I-O curves for a control cell (different cell) under voltage clamp, before and after tetanus. (**C**) Muscarine-treated cell under current clamp. Curves were constructed before (control) and 15 min after superfusion of 1 μM muscarine and then 15 min after tetanus. In this cell there was a small increase in the EPSP with muscarine, but no LTP was observed. (**D**) A similar experiment under voltage clamp (different cell). In this case 1 μM muscarine had little effect on the EPSC, and once again LTP was not obtained. In (C) and (D), muscarine was washed from the bath immediately after tetanus (*9*). (Points are mean of at least four determinations, and error bars represent SEM.)

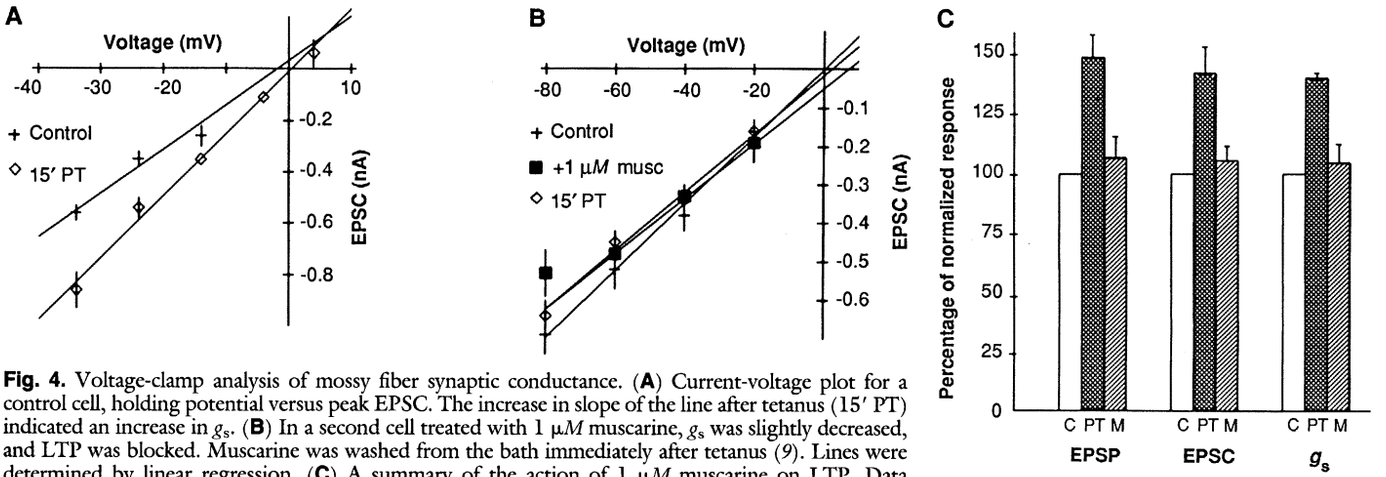

Fig. 4. Voltage-clamp analysis of mossy fiber synaptic conductance. (**A**) Current-voltage plot for a control cell, holding potential versus peak EPSC. The increase in slope of the line after tetanus (15′ PT) indicated an increase in g_s. (**B**) In a second cell treated with 1 μ*M* muscarine, g_s was slightly decreased, and LTP was blocked. Muscarine was washed from the bath immediately after tetanus (*9*). Lines were determined by linear regression. (**C**) A summary of the action of 1 μ*M* muscarine on LTP. Data normalized to before tetanus control. EPSP: 15′ PT, $n = 15$; 1 μ*M*, $n = 10$. EPSC: 15′ PT, $n = 11$; 1 μ*M*, $n = 7$. g_s: 15′ PT, $n = 4$; 1 μ*M*, $n = 5$. Abbreviations: C, control; PT, 15 min after tetanus; and M, +1 μ*M* muscarine.

An increased synaptic conductance (g_s) underlies LTP at the mossy fiber synapse, with no detectable change in passive membrane properties or synaptic reversal potential (*8*). We therefore tested the hypothesis that muscarine prevents this increase in g_s, rather than acting through other mechanisms such as an alteration of the synaptic reversal potential or an induced nonlinearity in the voltage dependence of the EPSC. The amplitude of the EPSC was determined at a range of holding potentials, and g_s was calculated from the slope of the current-voltage relation. In a control cell, g_s increased from 17 to 23 nS 15 min after tetanus, with little change in the reversal potential (Fig. 4A). In contrast, there was no significant increase in g_s in the cell tetanized in the presence of 1 μ*M* muscarine (Fig. 4B). In control cells the mean increase in g_s was +41 ± 2% ($n = 4$, four of four were greater than +20%), whereas in 1 μ*M* muscarine-treated cells the increase was significantly less, mean +5 ± 8% ($n = 5$, one of five was greater than +20%). Muscarine application itself produced a decrease in g_s of 12 ± 5% compared to control, but this was not significant. There was no significant shift in synaptic reversal potential for either group, either as a result of muscarine application or tetanization. A summary of the effects of muscarine on LTP induction is shown in Fig. 4C.

In the CA3 neuron, recurrent and commissural-associational inputs synapse on dendrites that are rich in NMDA receptors, whereas mossy fibers synapse proximal to the cell body in an area almost devoid of NMDA receptors (*14*). In view of the known APV sensitivity of LTP in the commissural-associational and Schaffer collateral pathways (*11, 15*), it is likely that LTP of recurrent synapses is also dependent on NMDA receptor activation, although this is difficult to test directly. Thus, the APV insensitivity of the LTP measured in this study was not surprising and, taken together with previous biophysical data (*6*), supports the notion that we are measuring LTP of the mossy fiber synapse.

We have demonstrated that the presence of muscarine during tetanus reduces the probability of induction and the amplitude of LTP at the mossy fiber–CA3 synapse, measured under current and voltage clamp. This action can be attributed to a block of the increase in excitatory g_s that normally accompanies LTP. Muscarine is not merely occluding LTP by depressing synaptic transmission because muscarine does not block LTP if added after the tetanus. The effects of muscarine were in contrast to those of norepinephrine, which enhances LTP at this same synapse (*7*). Because β-adrenergic receptor activation increases Ca^{2+} current in hippocampal neurons (*16*), and induction of LTP may depend on a rise in postsynaptic Ca^{2+} concentration (*17*) (although this has not been demonstrated for the mossy fiber synapse), norepinephrine might enhance LTP by increasing Ca^{2+} influx in the postsynaptic neuron. Muscarine appears to reduce Ca^{2+} currents in CA3 cells (*18*), suggesting that the action of muscarine on mossy fiber LTP might be mediated by a decrease in Ca^{2+} current in the CA3 cell, although other possibilities, including a presynaptic action of muscarine, cannot be ruled out.

If LTP is indeed a substrate for learning, then it is likely that its induction is carefully regulated. At the mossy fiber–CA3 synapse, a bidirectional control appears to be exerted by muscarinic and noradrenergic influences. Because LTP at the mossy fiber–CA3 synapse is APV insensitive, modulation at this synapse by neurotransmitters may be different from that observed at other synapses in the hippocampus. Although our finding that muscarine depresses LTP would not have been predicted on the basis of whole animal studies (*4*), it does support a role for cholinergic systems in memory, albeit a more complex role than previously thought.

REFERENCES AND NOTES

1. T. V. P. Bliss and T. Lømo, *J. Physiol. (London)* **232**, 331 (1973); P. Andersen and H. Wigström, in *Neurobiological Basis of Learning and Memory*, Y. Tsukod and B. W. Aghawoff, Eds. (Wiley, New York, 1980), pp. 17–47.
2. T. J. Teyler and P. Di Scenna, *Annu. Rev. Neurosci.* **10**, 131 (1987); L. R. Squire, *Science* **232**, 1612 (1986); D. S. Olton, in *Neurobiology of the Hippocampus*, W. Seifert, Ed. (Academic Press, New York, 1983), pp. 335–374.
3. C. C. D. Shute and P. R. Lewis, *Brain* **90**, 497 (1967).
4. J. O'Keefe and L. Nadel, *The Hippocampus as a Cognitive Map* (Clarendon Press, Oxford, 1978); J. A. Deutsch, *Science* **174**, 788 (1971); I. Q. Wishaw, W. T. O'Connor, S. B. Dunnet, *Behav. Brain Res.* **17**, 103 (1985).
5. R. T. Bartus, R. L. Dean III, B. Beer, A. S. Lippa, *Science* **217**, 408 (1982); P. J. Whitehouse *et al.*, *ibid.* **215**, 1237 (1982).
6. D. Johnston and T. H. Brown, *J. Neurophysiol.* **50**, 464 (1983); *ibid.*, p. 487.
7. W. F. Hopkins and D. Johnston, *Science* **226**, 350 (1984); *J. Neurophysiol.* **59**, 667 (1988).
8. G. Barrionuevo, S. R. Kelso, D. Johnston, T. H. Brown, *J. Neurophysiol.* **55**, 540 (1986).
9. The bath solution contained 124 m*M* NaCl, 3 m*M* KCl, 26 m*M* $NaHCO_3$, 3 m*M* $CaCl_2$, 3 m*M* $MgCl_2$, and 10 m*M* dextrose. Electrodes were filled with either 3*M* KCl or, to facilitate voltage clamping at depolarized membrane potentials, 2*M* CsCl or 3*M* KCl plus 100 m*M* QX314 (a Na^+ channel blocker). LTP was evoked by three trains of 100 stimuli (100 Hz), each separated by 5 s, applied once to each slice; test stimulation was at 0.2 Hz. Potentiation was expressed as the percentage change in peak EPSP or EPSC compared to normalized control values, measured just before tetanization. In most cases three stimulus intensities, around the lower third of the I-O curve, were compared and an average taken. The I-O curves or synaptic conductance plots were constructed every 15 min, and after tetanus four stimuli at 0.2 Hz were given at 5-min intervals to monitor the development of LTP. Drugs

were added to the perfusion medium and were present for at least 15 min before tetanic stimulation. In the case of muscarine, washing out was started immediately after the tetanus had been applied. In experiments involving APV, the drug was present during and after tetanus. Traces shown were digitized and represent an average of four to ten traces.
10. J. V. Halliwell and P. R. Adams, *Brain Res.* **250**, 71 (1982).
11. E. W. Harris and C. W. Cotman, *Neurosci. Lett.* **70**, 132 (1986).
12. J. A. Kauer and R. A. Nicoll, in *Synaptic Plasticity in the Hippocampus*, H. I. Haas and G. Buzsaki, Eds. (Springer-Verlag, Berlin, 1988), pp. 65–66.
13. R. J. Valentino and R. Dingledine, *J. Neurosci.* **1**, 784 (1981).
14. R. Lorente de Nó, *J. Psychol. Neurol.* **46**, 113 (1934); D. I. Gottlieb and M. Cowan, *J. Comp. Neurol.* **149**, 393 (1973); D. T. Monaghan and C. W. Cotman, *J. Neurosci.* **5**, 2909 (1985).
15. G. L. Collingridge, S. J. Kehl, H. McLennan, *J. Physiol. (London)* **334**, 33 (1983); H. Wigström and B. Gustafsson, *Neurosci. Lett.* **44**, 327 (1984).
16. R. Gray and D. Johnston, *Nature* **327**, 620 (1987); R. E. Fisher, R. Gray, D. Johnston, *Neurosci. Abstr.* **14**, 645 (1988).
17. G. Lynch, J. Larson, S. Kelso, G. Barrionuevo, F. Schottler, *Nature* **305**, 719 (1983).
18. B. H. Gähwiler and D. A. Brown, *Neurosci. Lett.* **76**, 301 (1987).
19. We thank F. Lebeda, J. Noebels, and P. Rutecki for comments on this manuscript. Supported by NIH grants HL31164 and NS11535, and the Air Force Office of Scientific Research grant AFOSR 85-0178. We also thank Astra Pharmaceutical Products for the gift of QX314.

28 March 1988; accepted 26 July 1988

Prenatal Tetrodotoxin Infusion Blocks Segregation of Retinogeniculate Afferents

CARLA J. SHATZ* AND MICHAEL P. STRYKER

In the adult mammalian visual system, ganglion cell axons from the two eyes are segregated from each other into separate layers within their principal target, the lateral geniculate nucleus. The involvement of spontaneously generated action potential activity in the process of segregation was investigated during the fetal period in which segregation normally occurs in the cat, between embryonic day 45 (E45) and birth (E65). Tetrodotoxin, which blocks the voltage-sensitive sodium channel, was used to prevent action potentials. Fetuses received continuous intracranial infusions of tetrodotoxin from osmotic minipumps implanted in utero on E42. After a 2-week infusion, intraocular injections of anterograde tracers revealed that tetrodotoxin prevented segregation. The contralateral projection filled the lateral geniculate nucleus uniformly, and the ipsilateral projection expanded to occupy most of what would normally be contralaterally innervated layer A. Thus, in the fetus, long before the onset of vision, spontaneous action potential activity is likely to be present in the visual system and to contribute to the segregation of the retinogeniculate pathway.

DURING THE DEVELOPMENT OF the vertebrate nervous system, the precise pattern of connections present in the adult often emerges from an initially diffuse set of connections. For example, in the adult mammalian visual system, inputs from the two eyes are segregated from each other into separate layers within the lateral geniculate nucleus (LGN) and into separate patches, the ocular dominance columns, within layer 4 of the primary visual cortex (*1*). However, the initial connections within each of these structures are not segregated. Geniculocortical axons serving the two eyes first make intermingled functional connections that drive cortical cells in layer 4 binocularly (*2*). When retinal ganglion cell axons from the two eyes first grow into the LGN, they are also intermixed (*3*). The eye-specific layers emerge gradually as the terminal arborizations of each eye's axons expand selectively in territory appropriate to the eye of origin, while branches located in inappropriate territory are lost (*4*).

Although the mechanisms responsible for this process of segregation are not well understood, several lines of evidence suggest that neuronal activity, such as patterns of action potentials and synaptic transmission, may play a role (*5*). Formation of ocular dominance columns in layer 4 of the cat's visual cortex, for example, can be prevented by eliminating retinal ganglion cell discharges with intraocular injections of the voltage-sensitive Na^+ channel blocker tetrodotoxin (TTX), but not by dark-rearing (*6*), which does not abolish the spontaneous activity of ganglion cells. These and other findings are consistent with the notion that spontaneous neuronal discharge is important for the refinement of neural connections.

We investigated the possibility that spontaneous activity plays a role in the refinement of connections prenatally even before vision is possible. The fact that retinal ganglion cell axons make ultrastructurally identifiable and functionally competent synapses within the LGN of the cat in utero (*7*) suggests that spontaneous neural activity could play a role in the formation of the eye-specific layers. To test this suggestion, we have infused TTX into the fetal brain to block action potentials during the time when ganglion cell axons from the two eyes would normally segregate from each other into layers.

Tetrodotoxin was infused continuously into the region of the brain above the optic chiasm beginning at embryonic day 42 (E42; gestation is 65 days), a time when axons from the two eyes are extensively intermixed within the LGN and have not yet begun to segregate (*8*). A total of ten animals was studied. Each fetus was exposed by Cesarian section for implantation of a cannula attached to an osmotic minipump containing either 300 μM TTX (eight animals) or a control citrate buffer vehicle solution (two additional animals) (*9*). [Chronic infusion of TTX at this concentration and rate blocks activity in the visual cortex and its afferents over an area of about 3 mm^3 (*10*).] The fetus was returned to the uterus for 2 weeks until E56, a time when the eye-specific layers are clearly evident in normal animals (*8*), and just before the minipumps ceased functioning. We used the anterograde transport of [^{3}H]leucine injected into one eye and horseradish peroxidase injected into the other to examine the effects of the treatment on the pattern of the retinogeniculate projection (*8*). The concentration of TTX in the fetal brains during the infusion was estimated to be between 0.1 and 1.0 μM in two of the TTX-treated animals by bioassay of the cerebrospinal fluid (CSF) removed by cisternal puncture at E49 (*11*) and was found to be between 0.1 and 1.0 μM, levels sufficient to block the compound action potential in neonatal rat optic nerve (*12*).

Despite the surgical manipulations and the presence of TTX during the 2-week infusion period, fetal growth was within the normal range. Between E42 and E58 the crown-rump length of fetuses normally increases from 57 to 68 mm to 100 to 120 mm (*8*); the TTX-treated animals at E56 were 105 to 110 mm (n = 3). In addition, the gross appearance of the brain, as revealed by examining histological sections

C. J. Shatz, Department of Neurobiology, Stanford University School of Medicine, Stanford, CA 94305.
M. P. Stryker, Department of Physiology, University of California, San Francisco, CA 94143.

*To whom correspondence should be addressed.