NT3 probe that included the entire region utilized as a probe for in situ hybridization. The differences we observed in both the pattern of tissue distribution and transcript size (BDNF, 3.5 and 1.6 kb; NT3, 1.4 kb) are consistent with a lack of cross-hybridization of the probes among members of the NGF family.
10. The localization of mRNA for BDNF, NT3, and NGF was compared in coronal sections of rat and mouse forebrain. The distribution of BDNF, NT3, and NGF mRNA in sections of rat and mouse brain through the hippocampus was examined in four separate experiments with tissue from 12 rats and 7 mice.
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13. Hybridizations were carried out as described (*5*). Templates for the sense and antisense probes were derived from a cDNA clone encoding for human BDNF (A. Rosenthal *et al.*, in preparation). The template and resulting probes were approximately 1 kb and contained ~600 bp of protein coding sequence and ~300 bp of 3′ untranslated sequence. On slot blots, this probe gave no detectable hybridization to RNA encoding for human NGF or NT3 at concentrations of these RNAs in 20-fold excess over that required for a hybridization signal to BDNF.
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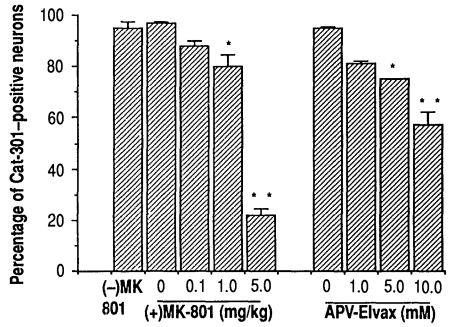

Fig. 1. MK-801 and APV inhibit Cat-301 immunoreactivity on sciatic motor neurons. Sample preparation as in Table 1. Scheffé's multiple-range test for comparisons within several experimental groups was used to show differences from vehicle-treated animals (*, $P < 0.05$; **, $P < 0.01$). The percentage of Cat-301–positive neurons from (–)MK-801–treated animals did not differ significantly from that of saline-injected controls.

Induction of a Neuronal Proteoglycan by the NMDA Receptor in the Developing Spinal Cord

ROBERT G. KALB* AND SUSAN HOCKFIELD

Activation of the *N*-methyl-D-aspartate (NMDA) subclass of glutamate receptors is a critical step in the selection of appropriate synaptic connections in the developing visual systems of cat and frog. Activity-dependent development of mammalian motor neurons was shown to be similarly mediated by activation of the NMDA receptor. The expression of the Cat-301 proteoglycan on motor neurons was developmentally regulated and could be specifically inhibited by blockade of the NMDA receptor at the spinal segmental level. In the adult, Cat-301 immunoreactivity on motor neurons was not diminished by NMDA receptor blockade. The NMDA receptor may regulate the expression of a class of neuronal proteins (of which Cat-301 is one example) that underlie the morphological and physiological features of activity-dependent development.

NEURONAL ACTIVITY IN THE EARLY postnatal period can have long-lasting effects on both the anatomy and physiology of neurons in the mammalian central nervous system (CNS). In many systems, activity-dependent effects on neuronal phenotype occur during circumscribed periods in early postnatal development, called critical or sensitive periods (*1*). In the cat visual cortex, activity-dependent acquisition of mature neuronal properties has been suggested to involve activation of the NMDA subclass of glutamate receptors (*2*). The molecular events that follow activation of the NMDA receptor and lead to alterations in neuronal phenotype are only beginning to be understood, but one hypothesis suggests that NMDA receptor activation leads to changes in the expression of neuronal proteins.

The expression of the neuronal cell surface proteoglycan recognized by monoclonal antibody Cat-301 (*3*) is regulated by neuronal activity in the early postnatal period. Visual deprivation of neonatal cats leads to a marked diminution of Cat-301 expression in the deprived layers of the dorsal lateral geniculate nucleus and in the visual cortex, whereas deprivation in adult cats has no effect on Cat-301 expression (*4*). Similarly, deprivation of normal patterns of neuromuscular activity in neonatal hamsters leads to a marked reduction in the expression of the Cat-301 proteoglycan on spinal cord motor neurons, whereas deprivation in adults does not (*5, 6*). The perisynaptic location (*7*) and developmental regulation (*4–6*) of the Cat-301 proteoglycan suggest that the antigen might be involved in the selection or stabilization of the mature set of synapses on motor neurons.

The expression of the Cat-301 antigen on motor neurons requires input from several sources (segmental and suprasegmental) in early life. At birth, hamster spinal motor neurons do not express the Cat-301 antigen; adult levels of expression are reached by the end of the second postnatal week (*5*). Disrupting sciatic motor neuron activity in neonates by nerve crush (*5*), dorsal rhizotomy (*6*), or thoracic cordotomy (*5*) at postnatal day 7 (P7) (before the onset of normal Cat-301 expression) inhibits the development of Cat-301 immunoreactivity on motor neurons. When adult animals are subjected to the same lesions, no effect on Cat-301 expression is seen. These studies provide molecular evidence for activity-dependent development of motor neurons.

Large-diameter primary afferents are required for normal expression of Cat-301 (*6*), and some of these are glutamatergic (*8*). Glutamate is considered the primary excitatory neurotransmitter in the spinal cord, and polysynaptic activation of motor neurons involves activation of the NMDA receptor (*9, 10*). The established role of NMDA receptors in activity-dependent development in other systems led us to explore the possibility that the NMDA receptor might also play a role in the maturation of spinal motor neurons, as assayed by Cat-301 expression.

As a first step in evaluating the effects of NMDA receptor blockade on the expression of Cat-301 immunoreactivity, we employed the lipophilic, noncompetitive NMDA receptor antagonist, MK-801. Animals received intraperitoneal injections of MK-801 daily from P7 to P21 at a range of doses (0.1 to 5.0 mg per kilogram of body weight). At first, animals appeared intoxicated, but

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

*To whom correspondence should be addressed.

Table 1. NMDA receptor antagonists reduce Cat-301 expression on motor neurons. Breeding and handling of hamsters has been described (*5, 6*). NMDA receptor antagonists were administered from P7 to P21 either by daily intraperitoneal injection (MK-801) or by Elvax implants (APV). Sciatic motor neurons were retrogradely labeled at P19 by sciatic nerve injection of 1 μl of 1% Fast Blue on P19. Preparation of tissue and immunohistochemical methods were as described (*5, 6*) and were carried out in parallel for control and experimental animals. A minimum of 300 retrogradely labeled cells from three animals from separate litters were counted. Values are means ± SEM. (−)MK-801 is the inactive stereoisomer.

NMDA receptor antagonist	Cat-301–positive neurons (%)
Neonate	
MK-801, 5.0 mg/kg	22 ± 2.4**
MK-801, 1.0 mg/kg	80 ± 4.5*
MK-801, 0.1 mg/kg	88 ± 2.0
Saline	97 ± 0.7
(−)MK-801, 5.0 mg/kg	95 ± 2.3
Adult	
MK-801, 10.0 mg/kg	96 ± 0.5
Saline	95 ± 0.5
Neonate	
Lumbar APV-Elvax, 10.0 mM	57 ± 5.1**
Lumbar APV-Elvax, 5.0 mM	75 ± 0.3*
Lumbar APV-Elvax, 1.0 mM	81 ± 1.2
Lumbar saline-Elvax	95 ± 0.3
Cervical APV-Elvax, 10.0 mM	84 ± 2.9†
Cervical saline-Elvax	96 ± 0.8
Cortical APV-Elvax, 10.0 mM	95 ± 1.1
Adult	
Lumbar APV-Elvax 10.0 mM	98 ± 0.3
Lumbar saline-Elvax	97 ± 0.3

*$P < 0.05$, **$P < 0.01$, Scheffé's multiple-range test for comparisons within several experimental groups, difference from vehicle-treated animal. †Significant difference between lumbar and cervical APV implants at $P < 0.05$.

within 1 hour neither behavioral changes nor weakness distinguished them from controls. We examined the effect of MK-801 on a defined population of neurons, the sciatic motor neuron pool, identified by retrograde labeling with Fast Blue. Daily MK-801 injections from P7 to P21 inhibited the expression of Cat-301 on sciatic motor neurons in a dose-dependent, stereospecific manner (Table 1 and Figs. 1 and 2).

To determine whether the sensitivity of Cat-301 expression to NMDA receptor inactivation was temporally restricted, we administered MK-801 at 5.0 or 10.0 mg/kg to adult animals daily for 14 days, and Cat-301 expression on sciatic motor neurons was assayed. In these animals, 96% of sciatic motor neurons were Cat-301–positive, and this value did not differ significantly from saline-injected controls where 95% of neurons were Cat-301–positive (Table 1 and Fig. 2). Thus, in contrast to our findings in the neonate, expression of Cat-301 on adult motor neurons appears to be independent of activation of the NMDA receptor. The differing effect of MK-801 on Cat-301 expression in neonates and adults suggests the existence of a circumscribed period in motor neuron development during which activation of the NMDA receptor is required for normal maturation.

To control for nonspecific effects of MK-801, we also assayed for expression of other neuronal proteins. Two other motor neuron antigens that are recognized by monoclonal antibodies Rat-302 and Rat-303 (*11*) are also initially expressed during the first two postnatal weeks (*5*). In MK-801–treated neonates that show the reduction in Cat-301 expression, 99% of sciatic motor neurons continue to express the antigens identified by Rat-302 and Rat-303 (Fig. 2). These results indicate that the inhibition of Cat-301 expression by MK-801 does not reflect a general impairment of all protein synthesis, but rather a reduction in the expression of a specific molecular species.

MK-801 may bind to sites in addition to the NMDA receptor (*12*). To control for the possibility that the observed reduction in Cat-301 immunoreactivity by MK-801 might be mediated by non-NMDA binding sites, we examined the effect of a second NMDA receptor antagonist on Cat-301 expression. Aminophosphonovaleric acid (APV) is a nonlipophilic, competitive NMDA receptor antagonist that binds at a site distinct from the MK-801 binding site on the NMDA receptor. Unlike MK-801, systemically administered APV does not cross the blood-brain barrier, so APV was administered by direct application at the lumbar enlargement. Slow, continuous release of APV was accomplished by incorporating APV into the polymer Elvax (*13, 14*) and placing a slice (1 mm by 2 mm by 50 μm) of Elvax over the lumbar enlargement, which was exposed by a laminectomy. Implants containing APV or saline were made at P7, and Cat-301 expression on sciatic motor neurons was assayed at P21. Only 57% of sciatic motor neurons were Cat-301–positive in animals receiving an implant containing 10.0 mM APV (Table 1 and Figs. 1 and 3). In contrast, 95% of sciatic motor neurons were Cat-301–positive in saline-implanted controls. Implants

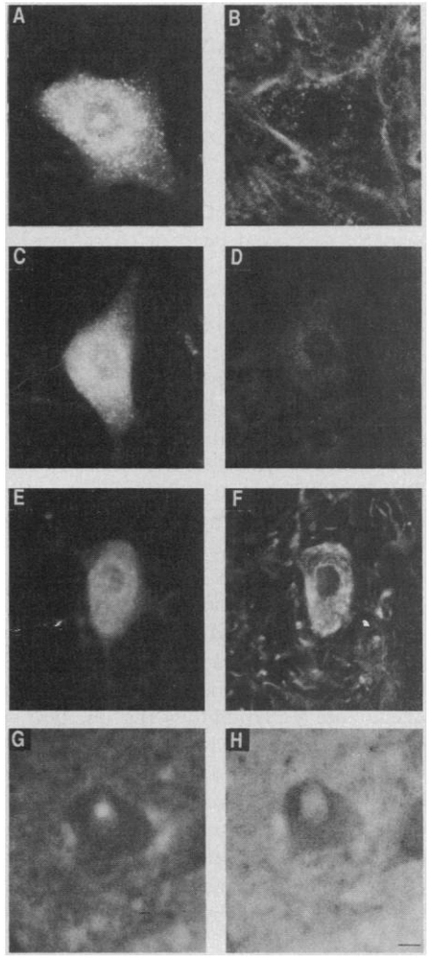

Fig. 2. MK-801 selectively inhibits Cat-301 staining of motor neurons from neonatal (C and D) but not adult (A and B) hamsters. All animals received MK-801 from P7 to P21. Motor neurons were identified with the retrograde tracer Fast Blue and viewed under fluorescent optics (A, C, E, and G). Antibody labeling was carried out with antibodies to mouse immunoglobulin G conjugated with fluorescein isothiocyanate (FITC) (B, D, and F) or horseradish peroxidase (HRP). (**A**) A Fast Blue–labeled motor neuron identified in the lumbar cord of an adult hamster after administration of MK-801 (10.0 mg/kg) for 14 days. (**B**) The same field as in (A) visualized for Cat-301 under FITC optics. Intense staining is visible around the perimeter of the neuronal cell body and proximal dendrites. The intracellular punctate staining is nonspecific and is due to lipofuscin pigment which fluoresces at this wavelength. It can be distinguished by color from the specific antibody staining (orange versus green) and is present in sections incubated in the absence of primary antibody. (**C**) A retrogradely labeled motor neuron from a hamster on P21 that had been administered MK-801 (5.0 mg/kg) from P7 to P21. (**D**) The same field as in (C) visualized for Cat-301 immunoreactivity shows no surface-associated staining. (**E**) Retrogradely labeled motor neuron from a hamster on P21 that had been treated daily with MK-801 (5.0 mg/kg) from P7 to P21. (**F**) The same field as in (E) demonstrates that this neuron is positive with a control antibody, Rat-303. (**G**) Retrogradely labeled motor neuron from a hamster on P21 that had been treated daily with MK-801 (5.0 mg/kg) from P7 to P21. (**H**) The same field as in (G) demonstrates staining with another control antibody, Rat-302. Scale bar, 8.5 μm.

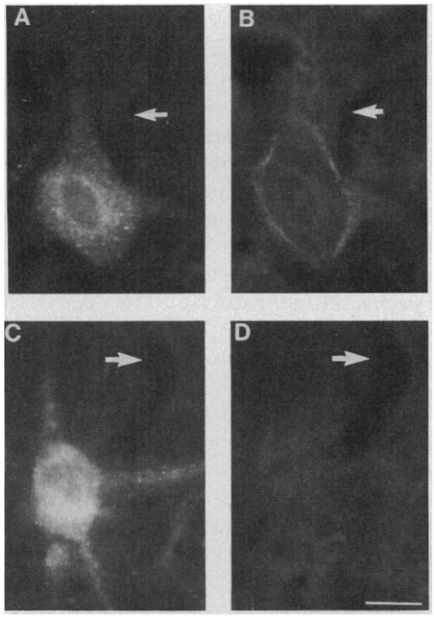

Fig. 3. Cat-301 staining is selectively reduced on motor neurons from neonatal (C and D) but not adult (A and B) hamsters treated with APV. Arrows denote a reference blood vessel in (A) and (B) and in (C) and (D). (**A**) A Fast Blue–labeled motor neuron from the lumbar cord of an adult hamster implanted with APV over the lumbar enlargement for 14 days. (**B**) The same field as in (A) viewed under FITC optics to visualize Cat-301 immunoreactivity. (**C**) A Fast Blue–labeled motor neuron from a hamster on P21 that had been implanted with APV (10.0 mM) over the lumbar enlargement at P7 for 14 days. (**D**) The same field as in (C) viewed under FITC optics shows no Cat-301 staining of the Fast Blue–positive motor neuron. Scale bar, 20 μm.

containing a range of APV concentrations produced a dose-dependent effect of APV on the development of Cat-301 immunoreactivity (Table 1 and Fig. 1). This reduction in Cat-301 immunoreactivity was molecularly specific, since the two other motor neuron antigens were not reduced by APV. Furthermore, the sensitivity to APV was developmentally regulated, since APV implants in adult animals had no effect on Cat-301, Rat-302, or Rat-303 expression when compared to saline-implanted controls (Fig. 3).

Our results indicate that the onset of Cat-301 expression on motor neurons requires activation of the NMDA receptor in early life. The precise neuronal circuitry subserving these effects cannot yet be determined unequivocally. To address the question of where in the neuroaxis the NMDA antagonists are operating, we compared the effects of implants placed over the lumbar enlargement, the motor cortex, and the cervicomedullary junction (in the cisterna magnum, an intermediate location). We reasoned that the closer the source of APV was to its site of action, the more potent it would be in regulating Cat-301 expression on motor neurons. When APV (10.0 mM) was implanted at the lumbar enlargement, only 54% of sciatic motor neurons were Cat-301–positive. When an implant (10.0 mM APV) was placed in the cisterna magnum, 84% of sciatic motor neurons were Cat-301–positive (saline implants had no effect: 96% Cat-301–positive). When 10.0 mM APV was implanted over the contralateral sensorimotor cortex, 95% of sciatic motor neurons were Cat-301–positive (Table 1). The effects of APV implants at the cervicomedullary junction and sensorimotor cortex were statistically significantly different from the lumbar implants ($P < 0.01$). These results suggest that the site of action of the NMDA antagonists on motor neuron development is at the spinal segmental level. The identity of the cell class within the neonatal spinal segmental unit that bears NMDA receptors is, at present, unknown.

Much of our understanding of the properties of the NMDA receptor comes from the study of long-term potentiation (LTP) in the hippocampus, a phenomenon considered to be a physiological correlate of learning and memory (*15, 16*). NMDA receptor activation requires coactivation of pre- and postsynaptic elements. The presynaptic inputs may arise from multiple sources (associativity) as long as they are coordinated such that a sufficient number are simultaneously active (cooperativity) to depolarize the postsynaptic cell (and thereby activate the NMDA receptor). These features of NMDA-mediated, long-lasting alterations in neuronal properties may have a parallel in the neuromuscular system: input from both descending (supraspinal) afferents and large diameter primary afferents are necessary for the expression of Cat-301 on motor neurons (*5, 6*). Our results suggest that convergence of descending and primary afferent inputs at the segmental level leads to the coherent activity necessary for activation of NMDA receptors and the normal maturation of motor neurons. The period of sensitivity of Cat-301 expression to NMDA receptor antagonism correlates with the period of acquisition of the mature properties of the segmental neuromuscular unit, such as synapse elimination from muscles (*17*) and from motor neurons (*18*). The present results suggest that long-lasting changes in the molecular, anatomical, and physiological properties of neurons as a consequence of use or activity are a general property in neuronal development.

It has been suggested that the effects of NMDA receptor activation on long-term changes in neuronal properties are mediated by changes in protein expression (*16, 19*). The results described here demonstrate that the expression of the Cat-301 surface-associated (*7*) chondroitin sulfate proteoglycan (*3*) is regulated by NMDA receptor activation. The Cat-301 proteoglycan may be one example of a class of proteins regulated by NMDA receptor activation, which underlie the structural and functional features of activity-dependent neuronal maturation.

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