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The Development of Sensorimotor Synaptic Connections in the Lumbosacral Cord of the Chick Embryo

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We have examined the development of synaptic connections between afferents and motoneurons in the lumbosacral spinal cord of the chick embryo betweeen stages 28 and 39. The central projection of afferents was visualized following injection of dorsal root ganglia with HRP. Afferent fibers first entered the dorsal gray matter between stages 29 and 31. They grew in a ventrolateral direction, reaching motoneuron dendrites by stage 32. Quantitative analysis of axon numbers suggested that individual axons did not begin to branch extensively until they approached the lateral motor column at stage 36.

Connectivity between afferents and motoneurons was assessed by stimulating dorsal roots or nerves supplying the femorotibialis muscle and recording the resulting motoneuron synaptic potentials intracellularly or from the cut ventral roots. At stages 37–39, low-intensity stimulation produced a short-latency positive potential that was followed at higher stimulus currents by slower positive potentials. All of these potentials were abolished in solutions that block chemical synaptic transmission (zero Ca^{2+}/2 mM Mn^{2+}). The early potential, which includes the monosynaptic EPSP produced by muscle afferents, persisted in the presence of the *N*-methyl-D-aspartate antagonist, 2-amino-5-phosphonovaleric acid (APV), but was largely eliminated by the more general excitatory amino acid antagonist, kynurenic acid. Therefore, in the chick, as in other species, a glutamate-like transmitter appears to be released at the synapses between muscle afferents and motoneurons. The APV-resistant potential was reduced in amplitude during bath application of the glycine and GABA antagonists, strychnine and picrotoxin, suggesting that it was composed of depolarizing inhibitory as well as excitatory components at these stages.

The monosynaptic EPSP could be recorded in ventral roots as early as stages 32–33, when muscle afferents first grew into the vicinity of motoneuron dendrites. The EPSP in these young embryos was unaffected by picrotoxin and strychnine, but responded to APV and kynurenate in a manner similar to that at later stages. Between stages 28 and 32, only long-latency, slowly rising potentials could be evoked in the ventral roots by afferent activation. These potentials were abolished by superfusion with zero Ca^{2+}/2 mM Mn^{2+}, APV, or kynurenic acid, and could be revealed before stage 31 only by removing Mg^{2+} from the bath.

The early appearance of synapses between muscle afferents and motoneurons in the chick at a time when both cell populations are making peripheral connections and decreasing in number through cell death raises the possibility that these processes may be important in regulating the formation of sensorimotor connections in this species.

The monosynaptic reflex involving Ia muscle afferents and α-motoneurons represents one of the most extensively studied pathways in the vertebrate spinal cord (for reviews, see Burke and Rudomin, 1977; Redman, 1979). Despite our knowledge about the structure and function of this pathway in adults, relatively little is known about the mechanisms controlling its formation during development. Early studies documented the growth of afferent collaterals into the spinal gray matter and the onset of reflexive behavior in a number of species (Windle, 1934; Windle and Orr, 1934; Windle and Baxter, 1936; Visintini and Levi-Montalcini, 1939; Levi-Montalcini and Levi, 1943). More recently, the use of precise anatomical labeling methods and electrophysiological recording has made it possible to follow the early development of specific reflex pathways at the cellular level. Of the investigations that have focused on the ontogeny of the Ia pathway, only those in the bullfrog (Frank and Westerfield, 1983) and the rat (Saito, 1979; Kudo and Yamada, 1987) have examined in detail the properties of direct sensorimotor connections at the time those connections first develop.

Some evidence suggests that the mechanisms controlling the formation of sensorimotor synapses might differ among species. In the bullfrog, muscle afferents appear to establish appropriate synaptic contacts with motoneurons *de novo* without substantial error (Frank and Westerfield, 1983). In contrast, Saito (1979) demonstrated that intersegmental reflexes evoked by stimulating dorsal root afferents in the rat were more widespread in the fetus than in the adult, although he did not examine the pattern of connectivity between afferents from particular muscles and different motoneuron pools. Further support for the possibility of interspecies differences in the regulation of sensorimotor connectivity is provided by the results of experimental manipulations in the frog and chick. While bullfrog motoneurons deprived of their normal afferent inputs by prior deletion of a dorsal root ganglion receive foreign synapses from an adjacent ganglion (Frank and Westerfield, 1982b), chick motoneurons are permanently deprived of their afferent inputs by partial neural crest removal (Eide et al., 1982).

In order to learn more about the mechanisms governing the formation of sensorimotor connections, we have examined the

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development, physiology, and pharmacology of afferent projections to motoneurons in the lumbosacral spinal cord of the chick embryo. The chick embryo is particularly suitable for developmental studies of sensory connectivity because of its brief incubation, its amenability to chronic surgical and pharmacological manipulations, and the wealth of accumulated information about the development of its spinal afferents and efferents (Windle and Orr, 1934; Oppenheim et al., 1975; Hamburger, 1976; Hamburger et al., 1981; Honig, 1982). Previous descriptions of the ontogeny of the muscle afferent–motoneuron pathway in the chick have been behavioral (Visintini and Levi-Montalcini, 1939) or anatomical (Johnson et al., 1986), or have concentrated on the relatively mature connections that are established just before hatching occurs (Eide et al., 1982). Our results extend those observations and indicate that the first direct, functional contacts between afferents from the femorotibialis muscle and motoneurons form early in embryonic development, before cell death in these neuronal populations is complete. Some of our findings have been published in abstract form (Koebbe and O'Donovan, 1985, 1987; Lee and O'Donovan, 1987).

Materials and Methods

All experiments were performed using White Leghorn chicken embryos obtained from a local supplier (Welp) and incubated at 37°C in a forced-draft incubator. Embryos were staged according to the criteria of Hamburger and Hamilton (1951).

Anatomical demonstration of afferent projections to lumbosacral motoneurons. Embryos were removed from their shells, decapitated, and transferred to a dissection dish continuously superfused at a rate of 10–30 ml/min with oxygenated (95% O_2/5% CO_2) Tyrode's solution at room temperature (21–24°C). The viscera were removed, a ventral laminectomy was performed over the thoracic and lumbosacral (LS) segments of the spinal cord, and the ventral roots (VRs) and dorsal root ganglia (DRGs) of the anterior 3 LS segments were exposed and separated.

Afferents or motoneurons were labeled with a 5–40% (wt/vol) aqueous solution of HRP containing 3% lysophosphatidyl choline (Frank et al., 1980). To label afferents, this solution was pressure-injected into 1–3 DRGs with a glass micropipette. In some experiments, afferents were labeled on one side and contralateral motoneurons of the same segments were retrogradely labeled by injecting HRP into the VRs. Generally, each DRG and VR was injected several times. To reduce the possibility that adjacent, noninjected roots might take up HRP, we cut them close to the cord and washed the area thoroughly with Tyrode's solution. Following the injections, the bath temperature was raised to 29–31°C and the superfusion was continued for 5–7 hr.

Injected embryos were fixed for 24–48 hr in phosphate buffer (100 mM, pH 7.2) containing 2% glutaraldehyde or a mixture of 2% glutaraldehyde and 1% paraformaldehyde. Either 3,3′-diaminobenzidine tetrahydrochloride (DAB; Polysciences) or tetramethylbenzidine (TMB; Sigma) was used as a substrate for HRP.

When DAB served as the substrate, the tissue was rinsed several times in 50 mM Tris (pH 7.2) and then incubated in DAB in 100 mM Tris (1 mg/ml) for 2.5 hr at 4°C. Hydrogen peroxide was added to make a final concentration of 0.03%, and the reaction was allowed to proceed for 3–5 hr at room temperature. The tissue was then dehydrated in ethanol and xylene, embedded in paraffin, and sectioned transversely at 10 or 20 μm on a microtome.

In experiments where TMB was to be used as the chromogen, the fixed tissue was placed in 25% sucrose in phosphate buffer for 3–24 hr, frozen in O.C.T. compound (Miles Scientific), and sectioned at 40 μm on a cryostat or frozen-stage sliding microtome. Sections were reacted with TMB at room temperature by one of 2 procedures. In the first (Mesulam, 1978), the sections were incubated for 15–20 min in 10 mM acetate buffer (pH 3.3) containing 0.005% TMB and 0.1% sodium nitroferricyanide. Hydrogen peroxide was added (final concentration, approximately 0.01%), and the sections were reacted for 20–30 min. In the second procedure (Olucha et al., 1985), sections were incubated for 15–20 min in 100 mM phosphate buffer (pH 6.0) containing 0.005% TMB and 0.25% ammonium heptamolybdate. Hydrogen peroxide was added (final concentration, approximately 0.01%), and the reaction was monitored under a microscope and stopped when staining was judged to be optimal (usually at 15–30 min). This method resulted in reduced spicule formation, less tissue shrinkage, and lower background staining than the first method. Reacted sections were mounted on slides, cleared in ethanol and xylene, and coverslipped.

Labeled afferents and motoneurons were reconstructed in sections from the first and second lumbosacral segments (LS1 and LS2), using a computer-controlled tracing device mounted on a microscope. Reconstructed images were digitized at a resolution of 3 μm and drawn on a plotter (Hewlett-Packard 7475A). To quantify the dorsoventral growth of afferent axons and motoneuronal dendrites at different stages, a single transverse section showing the most extensive afferent or motoneuronal projection was selected from each embryo. That section was divided into 10–20 regions of equal width by a set of parallel, mediolateral lines, and the relative length of axons below or dendrites above each region was expressed as a percentile. Thus, 100% of the total length of afferent axons occurs below the dorsal roof of the cord and successively smaller fractions occur more ventrally, while the converse is true for motoneuronal dendrites. In addition, the number of afferent axons crossing each mediolateral line was counted in order to estimate the extent of axonal branching in each region.

Physiological and pharmacological techniques. The ventral surface of the spinal cord was exposed by the procedure described above, and the meninges and dura mater were removed. The cord was then transected longitudinally along the midline, and remnants of the vertebrae were extracted from between the spinal roots on one side (usually the left). The half of spinal cord on that side was lifted out of the vertebral canal, along with the LS dorsal roots (DRs), VRs, and selected hindlimb muscle nerves. This preparation was then pinned to a layer of Sylgard on the bottom of a 25 × 75 mm plexiglass chamber, through which oxygenated Tyrode's was superfused at 11 ml/min. All experiments were performed at room temperature.

In most of our experiments, we stimulated afferents by shocking one or more of the nerve branches innervating the femorotibialis muscle. We chose this muscle because its large nerves make a substantial afferent contribution to the spinal cord and are easy to identify and dissect, even in young embryos. In a few embryos, afferents were activated by shocking the DRs of LS2 or LS3. Stimuli were delivered with suction electrodes and consisted of 0.1–0.5 msec pulses of either polarity. The efficacy of these stimuli was monitored by recording the afferent volley in LS3 DR or LS3 DRG, using either suction electrodes (bandwidths, 0.1 Hz to 1 or 5 kHz) or tungsten microelectrodes whose tips were coated with platinum black (tip diameter, 25 μm, bandwidth 10 Hz to 5 kHz; Medical Systems).

Motoneuron responses to DR or femorotibialis nerve stimulation were recorded from the VR of LS2 or LS3 and intracellularly from LS3 motoneurons. We chose LS2 and LS3 because the majority of femorotibialis motoneurons are located in those segments (Landmesser, 1978; Hollyday, 1980), and because muscle afferents in other species usually project most strongly to motoneurons that innervate their own muscles (Eccles et al., 1957; Frank and Westerfield, 1982a). Recordings from the VRs were accomplished with tight-fitting suction electrodes and were filtered at 0.1 Hz to 1 or 5 kHz. At this bandwidth, motoneuron synaptic potentials, as well as action potentials, were preserved without significant distortion. Intracellular recordings from motoneurons, identified by antidromic activation from the VRs or muscle nerves, were made with glass microelectrodes filled with 2 M potassium citrate and having resistances of 90–130 MΩ. A motorized drive (Newport) was used to advance the microelectrodes into cells. Recordings were accepted only from cells in which the resting membrane potential stabilized at or below −40 mV within a few minutes after penetration, although the resting potential was not monitored throughout the duration of impalement in all motoneurons. Amplified signals were digitized at 10 kHz using a commercially available data-acquisition package (Computerscope, RC Electronics) run on an IBM XT and stored on a hard disk. Selected signals were reproduced on a graphics printer or a plotter with one of the data-conditioning programs included in that package or with another commercially available graphics program (PrintGraph, Lotus).

Normal Tyrode's had the following composition (mM): NaCl, 139; KCl, 2.9; $NaHCO_3$, 17; $CaCl_2$, 3; $MgCl_2$, 1; glucose, 12.2 (pH 7.4). Mg^{2+}-free saline was prepared by removing $MgCl_2$ from normal Tyrode's without substitution. The pharmacology of sensorimotor transmission

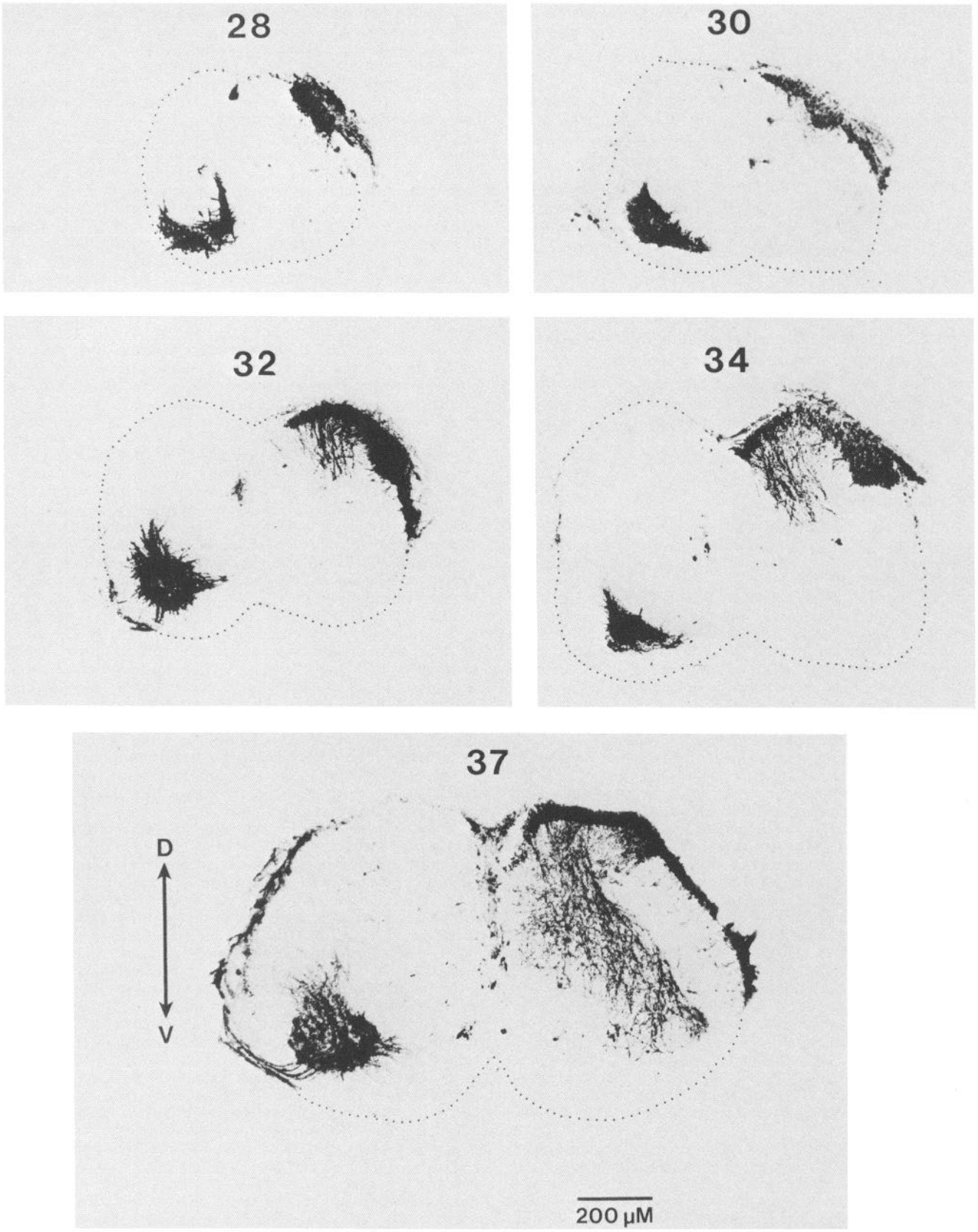

Figure 1. Photomicrographs of transverse sections of the lumbosacral spinal cord (LS1 or LS2) at several stages of development, illustrating the relative growth of afferents and motoneurons. HRP was injected into 1–3 DRGs on the *right side* and the corresponding VRs on the *left side*. All sections were cut at 40 μm and processed with TMB. Spinal cord boundaries are indicated by *dotted lines*.

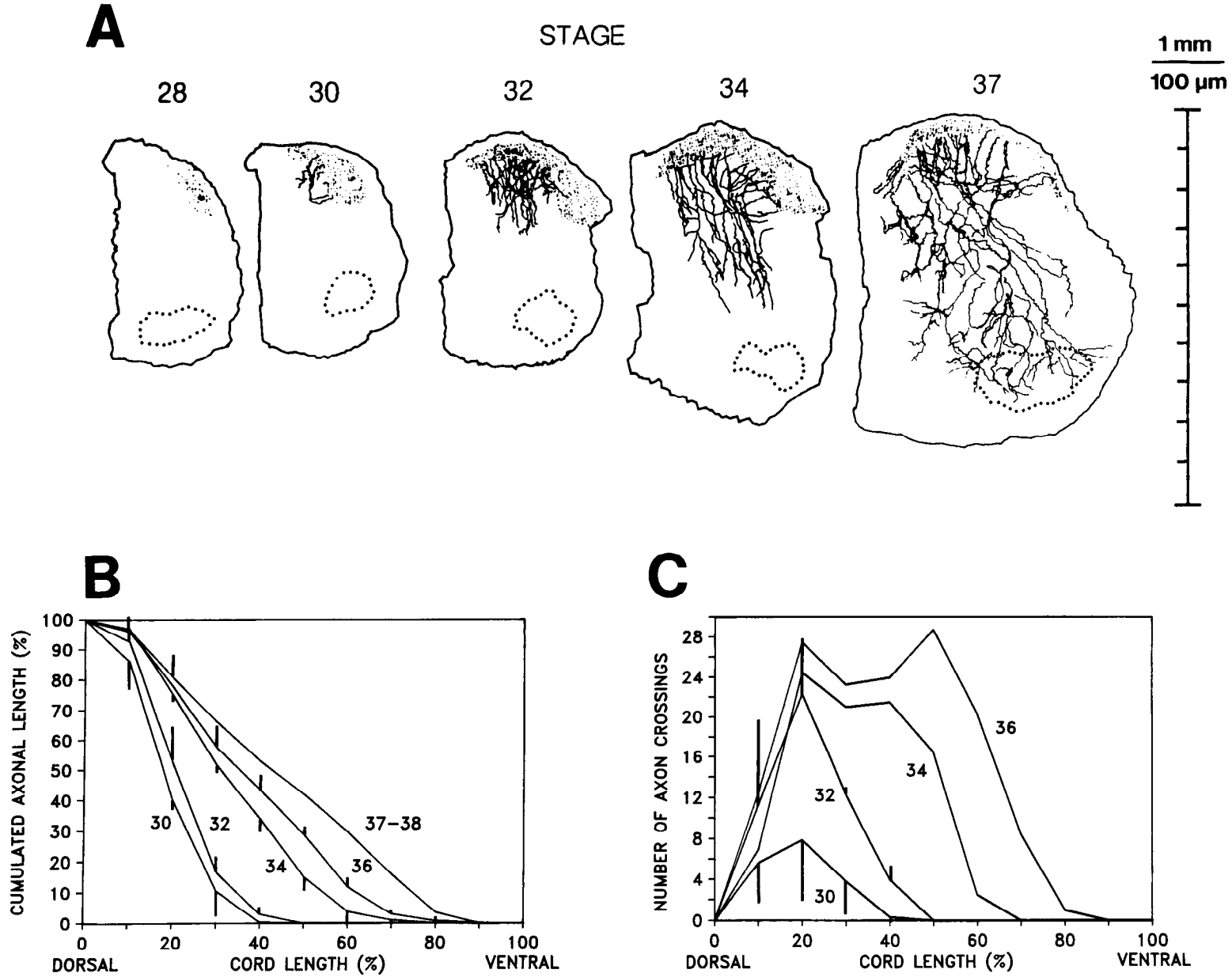

Figure 2. Quantification of the growth of afferents in the spinal cord. *A*, Computer reconstructions of afferent fibers in the right half of the cord at LS2, following injection of LS1–LS3 DRGs with HRP. Each reconstruction was drawn from a single 40 µm section that exhibited the most ventral afferent projection at that stage. Medial is at the *left* and dorsal is at the *top* in each section. The boundaries of the lateral motor column are indicated by *dotted lines*. *B*, The dorsoventral projection of afferent axons at different developmental stages. At each stage, the fraction of the cumulated length of afferent axons below successive 10% increments in the dorsoventral length of the cord is plotted against the cord length. *C*, Number of axon segments crossing successive 10% increments of the dorsoventral cord length. Data in *C* and *D* were obtained from 2–3 embryos at each stage and are plotted as mean ± SD (for $n > 3$).

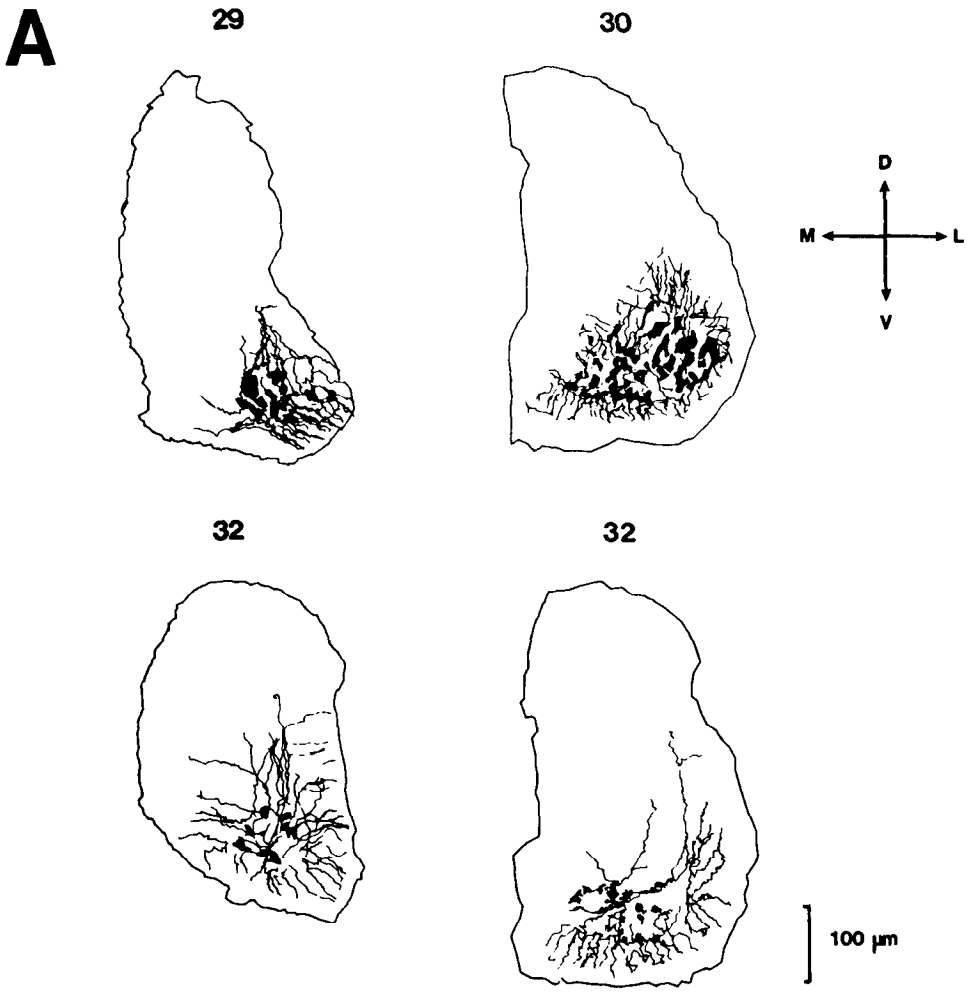

Figure 3. Quantification of the growth of motoneurons during stages 29–32. *A*, Computer reconstructions of motoneurons in LS1 and LS2 following injection of VRs with HRP. The 2 sections on the *left* were cut at 10 μm and processed using DAB as the chromogen; those on the *right* were cut at 40 μm and processed with TMB. Each reconstruction was drawn from a single section that showed the most dorsal dendritic extension with each chromogen. *B*, The ventrodorsal growth of motoneuronal dendrites at stages 29–30 and 32. At each group of stages, the fraction of the cumulated length of motoneuronal neurites (axons and dendrites) above successive 5% increments in the dorsoventral length of the cord is plotted against the cord length. Data were obtained from 3 embryos at each stage and are plotted as mean ± SD. Portions of the curves for stages 30 and 32 from Figure 2*B* have been reproduced to illustrate the development of overlap between afferents and motoneuronal dendrites during these stages.

was studied by superfusing the preparation with solutions of the following agents added to normal or Mg^{2+}-free Tyrode's: APV (200 μM; Sigma and Cambridge Research Biochemicals); kynurenic acid (1.25 mM; Sigma); picrotoxin (100 μM; Sigma); and strychnine (10 μM; Sigma). To block chemical synaptic transmission, $CaCl_2$ was replaced with 2 mM $MnCl_2$ in normal or Mg^{2+}-free Tyrode's. High-calcium/high-magnesium solution contained 12 mM Ca^{2+} and 5 mM Mg^{2+}; in 1 of 2 experiments in which this solution was used, [NaCl] was reduced to 119.5 mM to maintain normal osmolarity.

Results

Anatomical analysis of afferent and motoneuron development

The growth of afferent fibers into the spinal cord was examined by injecting HRP into the DRGs of the first 3 lumbosacral segments in 24 embryos between stages 28 and 37. In 15 of these embryos, the dendritic development of contralateral motoneurons in the same segments was also monitored, following HRP injections into the VRs. Three embryos were processed with DAB, 6 with TMB/nitroferricyanide, and 15 with TMB/heptamolybdate (see Materials and Methods). Although qualitatively similar results were obtained with all 3 methods, the TMB procedures proved more useful for quantitative measures because of their lower background staining and higher sensitivity. Representative photomicrographs of labeled afferents and motoneurons are illustrated in Figure 1, and reconstructions of sensory and motor projections are shown in Figures 2*A* and 3*A*, respectively.

In 2 embryos injected with HRP at stage 28, labeled sensory fibers had penetrated the spinal cord but appeared to be confined to a dorsolateral band of white matter. Approximately 1 d later, at stages 29–30 (4 embryos), sensory axons could be detected in the gray matter. At these stages, 90% of the cumulative length of labeled afferent axons occurred in the dorsal 30% of the cord (ca. 50 μm), and no axons extended below the dorsal 40% (Fig. 2*B*). The total number of labeled afferents within the gray matter increased substantially by stage 32, although more than 80% were still found in the dorsal third of the cord. As development proceeded the sensory projection continued to grow ventrolaterally so that, by stages 36–37, sensory axons were distributed widely in the spinal cord and had reached the lateral motor column in all 7 embryos examined.

Inspection of the afferent morphologies illustrated in Figures 1 and 2*A* suggests that sensory axons do not branch significantly until after stage 34. To quantify the degree of branching at different stages, we counted the number of axon segments that crossed mediolateral lines at 10% intervals along the dorsoventral axis of the cord (Fig. 2*C*). The number of axon crossings peaked in the dorsal 20% of the cord at all stages. At stages 30 and 32, this number declined ventrally, as would be expected if little branching occurred. Between stages 34 and 36, however, a second peak in the number of axon crossings appeared just below the dorsoventral midline of the cord, suggesting that individual axons began to branch there during that period of development. This area of branching coincides with the dorsal portion of the lateral motor column (Fig. 1).

The elaboration of motoneuronal dendrites at stages 29–30 and 32 was measured in a manner similar to that used to assess the growth of afferents (Fig. 3*B*). This measurement was not performed past stage 32 because we were unable to obtain consistent labeling of dendrites in older embryos. At stages 29–30, more than 80% of the cumulative dendritic length occurred in the ventral third of the cord, and there was no overlap between the territories occupied by afferents and motoneuronal dendrites. In contrast, 3 of 6 embryos at stage 32 contained dendrites of labeled motoneurons within the dorsal third of the gray matter, in the vicinity of the most ventrally projecting sensory axons. Therefore, the morphological basis for a monosynaptic connection between afferents and motoneurons is present by at least stage 32. While it is conceivable that undetected contacts between very small afferent terminals and dendrites exist prior to stage 32, sensorimotor synapses also become resolvable electrophysiologically at this stage (see below).

Physiological analysis of sensorimotor connectivity

Properties of sensorimotor connectivity in stage 37–39 embryos

In 15 embryos examined at stages 37–39, brief shocks of moderate intensity, delivered to a femorotibialis nerve branch or a DR in normal Tyrode's, elicited composite, positive-going potentials in LS2 or LS3 VR (Fig. 4*A*). With femorotibialis nerve stimulation, the earliest component of these potentials began approximately 5 msec after the arrival of the afferent volley in the corresponding DRG (range, 3.9–6.0 msec; 10 embryos). This component usually fused with potentials appearing at longer latencies, including large, cyclic potentials produced by the central pattern generator for rhythmic limb movements (O'Donovan and Landmesser, 1987). The early component was the first to appear as the stimulus intensity was gradually increased from levels that were subthreshold for afferent activation (Fig. 4*B*), and often its appearance could be correlated with that of the fastest afferent spikes in the DRG (not shown). A small, predominantly negative deflection coincident with the afferent volley in the DRG record typically preceded the positive-going potential in the VR trace at these stages (see Fig. 11). In most cases, this deflection probably represented the extracellular field potential of afferent spikes within the spinal cord, since similar potentials have been associated with afferent invasion of the spinal cord in cats (Munson and Sypert, 1979). However, in peparations where the recording electrode on the VR was very close to the DRG, the VR electrode may have picked up the afferent volley directly from the DRG. This latter possibility was especially likely in the smaller cords dissected from young embryos.

In order to determine whether the composite potentials recorded from the VRs accurately reflected the synaptic and spiking activity occurring in individual motoneurons, we recorded from LS3 VR and intracellularly from 31 LS3 motoneurons during stimulation of muscle nerves in 10 embryos. Antidromic spike amplitudes ranged from 22 to 70 mV (mean, 51 ± 12 mV; $n = 28$ cells) following VR or muscle nerve stimulation. Extracellular field potentials resulting from muscle afferent activation were not detectable. While most cells could be held for no more than approximately 10 min, in a few cases the impalements were stable for 3–4 hr, long enough to examine the effects of several changes in superfusate composition (see below).

In the vast majority of the cells we recorded from (26 of 31), femorotibialis nerve stimulation elicited a depolarization in the motoneuron that was very similar to the VR potential, with both early and later components when these were present in the VR (Fig. 5, *A–C*; also see Figs. 6–8*B*). The latency of the early PSP in these motoneurons was equal to that of the early component of the VR potential. The remaining 5 motoneurons were either unresponsive to stimulation of the femorotibialis nerve or were hyperpolarized by it (Fig. 5*D*). Two of these motoneurons, both in stage 39 embryos, were depolarized by stimulation

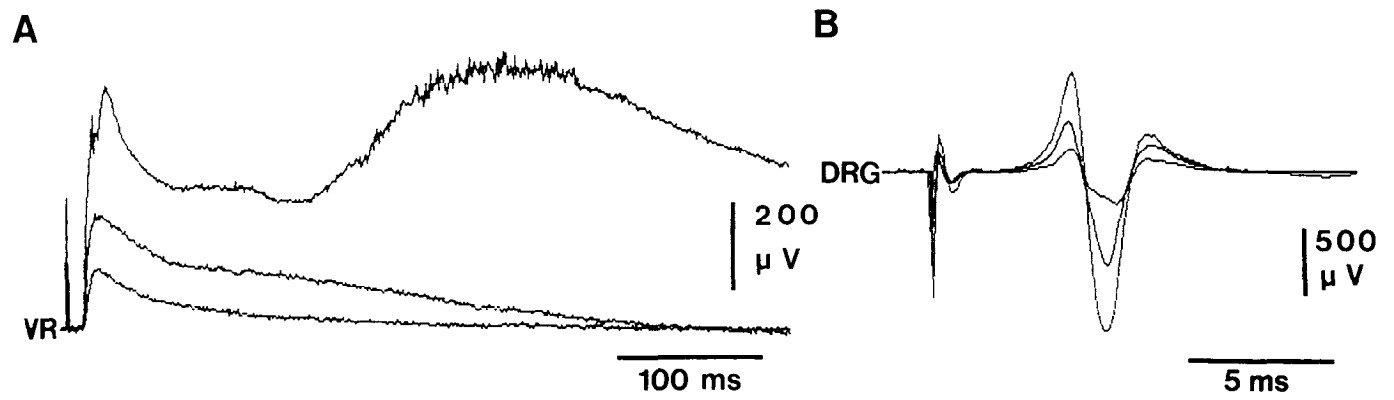

Figure 4. Gradual recruitment of the various components of muscle nerve-evoked VR potentials with increasing stimulus intensity. *A*, Recordings from LS3 VR in a stage 39 embryo during stimulation of a femorotibialis nerve branch in normal Tyrode's. At low stimulus intensity (*bottom*), only the early component was elicited. At an intermediate intensity (*middle*), this component increased in amplitude and began to merge with longer-latency potentials. High-intensity stimuli (*top*) evoked motoneuron spikes on the early component and later, during the first of several large potentials produced by the locomotor pattern generator. *B*, Recordings of the afferent volley produced in LS3 DRG by the stimuli delivered in *A*.

of the antagonistic sartorius nerve (Fig. 5*E*), indicating that at least some motoneurons respond differentially to activation of afferents from antagonistic muscles by this stage of development.

Several lines of evidence suggested that the shortest-latency component of the VR potentials included the monosynaptic EPSP generated in motoneurons by Ia muscle afferents. As shown above, the early component could be elicited in association with the firing of low-threshold, rapidly conducting sensory axons, which may belong to Ia afferents (Brinley, 1974). This component persisted in high-calcium/high-magnesium solutions that blocked later events (2 embryos; data not shown). In addition, its latency matched that of the earliest potentials recorded intracellularly in motoneurons and remained constant in each preparation, even during repetitive stimulation at 10–20 Hz.

Moreover, the reaction of the early VR potential to pharmacological manipulations was characteristic of the Ia EPSP in motoneurons of the newborn rat (Jahr and Yoshioka, 1986). In the presence of the *N*-methyl-D-aspartate (NMDA) antagonist, APV (200 μM; 8 embryos), the early component remained, while most or all of the later components were abolished (Fig. 6). Because of the fusion of the various components of the VR potential, it was impossible to determine precisely the time course and amplitude of the monosynaptic component in normal Tyrode's. Therefore, potentials obtained during drug administration were compared with the maximum amplitude attained during the initial 10 msec of the VR potential in normal Tyrode's. The APV-resistant potential had a time-to-peak of approximately 6–10 msec and a maximum amplitude that ranged from 68 to 96% of the amplitude of the early potential in normal saline. In contrast, the nonselective inhibitor of excitatory amino acid receptors, kynurenic acid (1.25 mM; 5 embryos), reduced the amplitude of the early component to 15–22% of its value in normal Tyrode's and eliminated later potentials (Fig. 6). In one embryo from which recordings were made simultaneously from LS3 VR and an LS3 motoneuron, the effects of APV and kynurenic acid on VR potentials were paralleled by similar changes in the motoneuron (Fig. 6). No recovery of the VR potential was seen when superfusion with these agents was maintained (up to 1 hr for APV and 40 min for kynurenic acid), but their actions were completely reversed upon return to saline lacking the drugs.

The presence of depolarizing IPSPs has been reported in motoneurons of the newborn rat (Takahashi, 1984; Jahr and Yoshioka, 1986) and the chick embryo (Velumian, 1984). To determine whether any part of the APV-resistant component of muscle nerve-evoked potentials in our preparations might be inhibitory, we examined the actions of picrotoxin and strychnine, blockers of GABA- and glycine-based IPSPs, respectively, on the potential isolated by APV (3 embryos, all at stage 39). In all cases, the addition of these inhibitory blockers reduced the amplitude of the later portion of the VR response, leaving a potential that appeared at the same latency as the early component. We believe this potential represents the monosynaptic EPSP. In one of these experiments, the effects recorded in the VR were corroborated by simultaneous, intracellular recordings from an LS3 motoneuron (Fig. 7). Subtraction of the traces obtained in APV, picrotoxin, and strychnine from those in APV alone yielded positive-going potentials in the motoneuron and the VR that began approximately 5 msec after the onset of the monosynaptic EPSP. These results imply that muscle nerve-evoked potentials isolated by APV were not purely monosynaptic in our preparations; they must have contained di- or perhaps polysynaptic potentials mediated by inhibitory transmitters.

Extracellular Mg^{2+} at concentrations above 10 μM has been shown to be an effective depressant of motoneuron depolarizations induced by NMDA and by afferent activation (Ault et al., 1980). This observation raised the possibility that some portion of the afferent input to motoneurons in our preparations might be inhibited in normal Tyrode's, which contained 1 mM Mg^{2+}. To address this question, we recorded VR potentials elicited by femorotibialis nerve stimulation in saline lacking Mg^{2+} (4 embryos). Changing the superfusate from normal to Mg^{2+}-free Tyrode's markedly enhanced the VR response, causing a larger underlying synaptic potential and a higher incidence of spiking (Fig. 8*A*). Furthermore, when the preparation was returned to normal Tyrode's after the exposure to Mg^{2+}-free saline, the VR potential was depressed below its original magnitude in normal Tyrode's (Fig. 8*A*). After approximately 1.5 hr in normal saline, the VR potential recovered partially, but was still smaller than the response evoked initially in normal Tyrode's. These solution changes had very little effect on the size of the afferent volley (Fig. 8*A*).

To determine whether alterations in $[Mg^{2+}]$ affected the mono-

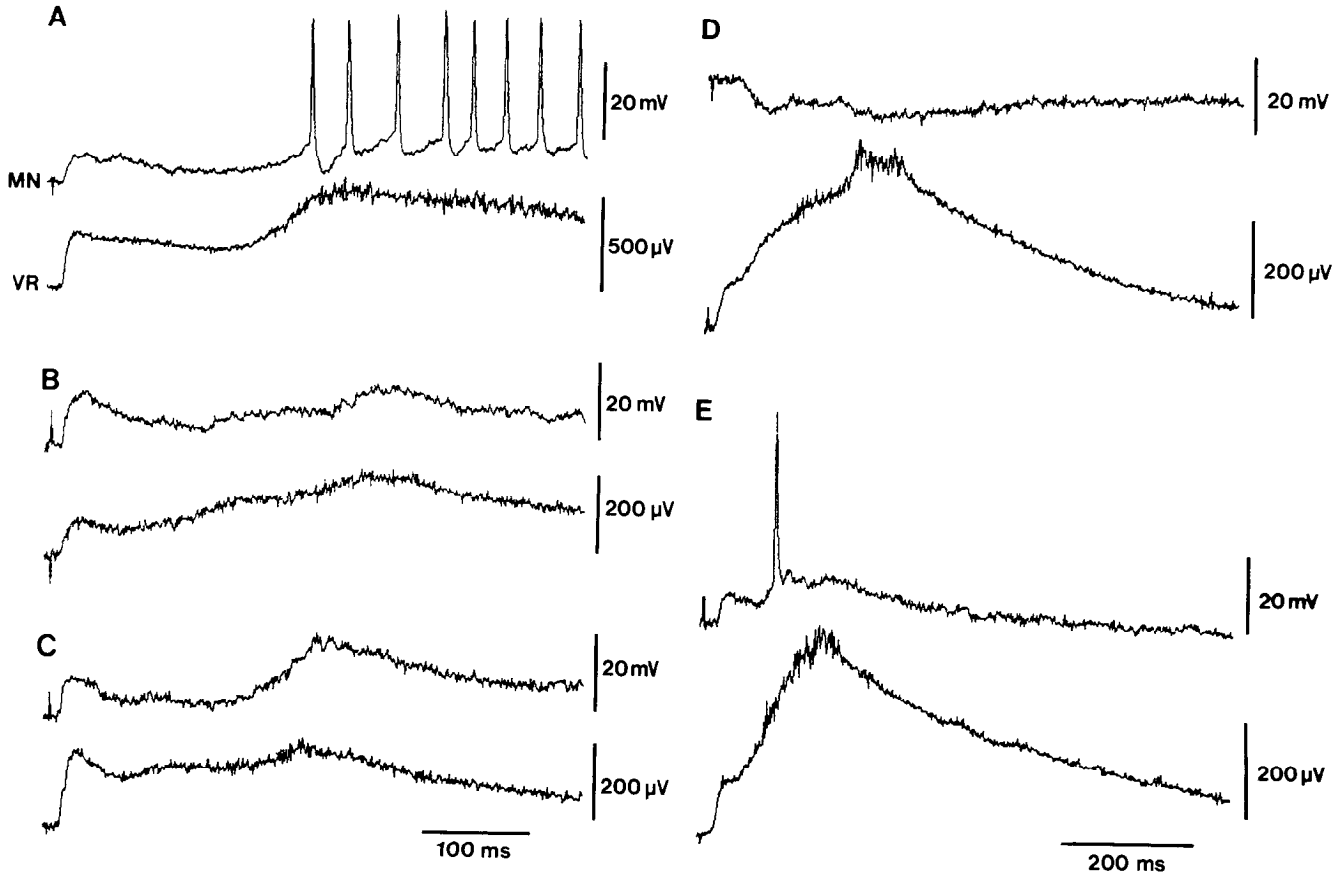

Figure 5. Correlation between nerve-evoked potentials recorded in VRs and motoneurons. Intracellular recordings from LS3 motoneurons (*MN*; *upper*) are paired with simultaneous recordings from LS3 VR (*lower*) in 3 embryos at stage 39. *A–C*, Three motoneurons that showed a synaptic response very similar to that elicited in the VR. All 3 were recorded in normal Tyrode's during stimulation of the same femorotibialis nerve branch. Records in *A* were from the same VR and motoneuron as are shown in Figure 6; those in *B* and *C* were obtained from another embryo. *D, E*, A motoneuron that was hyperpolarized by stimulation of the femorotibialis nerve (*D*) and depolarized by sartorius nerve stimulation (*E*). Both nerves produced positive-going potentials in the VR. The recordings were made in Mg^{2+}-free Tyrode's.

synaptic EPSP produced by muscle afferents, we isolated the EPSP in normal Tyrode's containing APV, picrotoxin, and strychnine, while recording from LS3 VR and an LS3 motoneuron (one embryo; Fig. 8*B*). Switching to Mg^{2+}-free Tyrode's containing these agents resulted in a slight increase in the size of the EPSP in the motoneuron but almost no change in the VR potential. These results suggest that most of the potentiation of sensorimotor transmission by Mg^{2+} removal occurs in polysynaptic pathways. The mechanisms through which Mg^{2+} might act in this system are considered in the Discussion.

Development of sensorimotor connectivity

The development of functional connections between afferents and motoneurons was examined by recording afferent-evoked potentials from LS2 or LS3 VR in 16 embryos at stages 28–35. In normal Tyrode's, early and later components of femorotibialis nerve-evoked VR potentials were demonstrable by stages 32–33 (1 of 2 embryos at stage 32, and 1 of 1 at stage 33; Fig. 9). Because of its small amplitude, the early component was difficult to resolve without signal-averaging at these stages. As in more mature embryos, the early component at stages 32–33 was strongly inhibited by 1.25 mM kynurenic acid (one embryo), but was largely unaffected by 200 µM APV, which abolished later potentials (2 embryos). The latency of the early component was comparable to that at stages 37–39, while its time-to-peak in APV (2–4 msec) was considerably shorter. In contrast to the potentials recorded at later stages, the APV-resistant component at stage 33 was not reduced by picrotoxin and strychnine (one embryo). By stages 34–35, the early component had increased in size, while retaining its sensitivity to kynurenic acid (one embryo) and its insensitivity to APV (2 embryos; Fig. 9). Although the amplitude of the early component at these intermediate stages was smaller than at stages 37–39, its latency and time-to-peak in APV were within the range of values for the later stages.

In one embryo examined at stage 31, the response in LS3 VR to femorotibialis nerve stimulation in normal Tyrode's consisted of a slowly rising positive potential preceded by a very small early potential (Fig. 9). The early potential, which was eliminated in Ca^{2+}-free, Mn^{2+}-containing saline, may have been the precursor of the early synaptic component seen at later stages. However, since we did not test its sensitivity to other pharmacological blockers, we cannot exclude the possibility that it represented a Ca^{2+}-sensitive part of the afferent volley picked up by the VR electrode.

Prior to stage 31, stimulation of femorotibialis nerves at intensities sufficient to elicit a maximal afferent volley in LS3 DRG failed to evoke any synaptic response in LS3 VR in normal Tyrode's (2 embryos at stage 30, 3 at stage 29, and 1 at stage 28; Fig. 9). Activation of afferents via DR or spinal nerve stim-

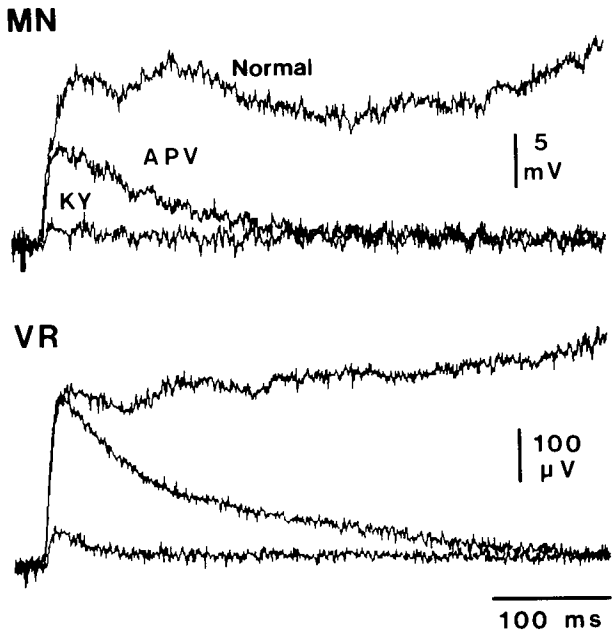

Figure 6. Sensitivity of muscle nerve-evoked VR potentials and motoneuron depolarizations to APV and kynurenic acid. Responses to stimulation of a femorotibialis nerve branch were recorded intracellularly in an LS3 motoneuron (*MN*; *top traces*) and in LS3 VR (*bottom traces*) in a stage 39 embryo. In normal Tyrode's, composite potentials were recorded in the VR and the motoneuron. Superfusion of the preparation with 200 μM APV preserved the earliest part of the synaptic response and abolished later components. Kynurenic acid (*KY*; 1.2 mM) greatly reduced the amplitude of the early response and completely eliminated later components.

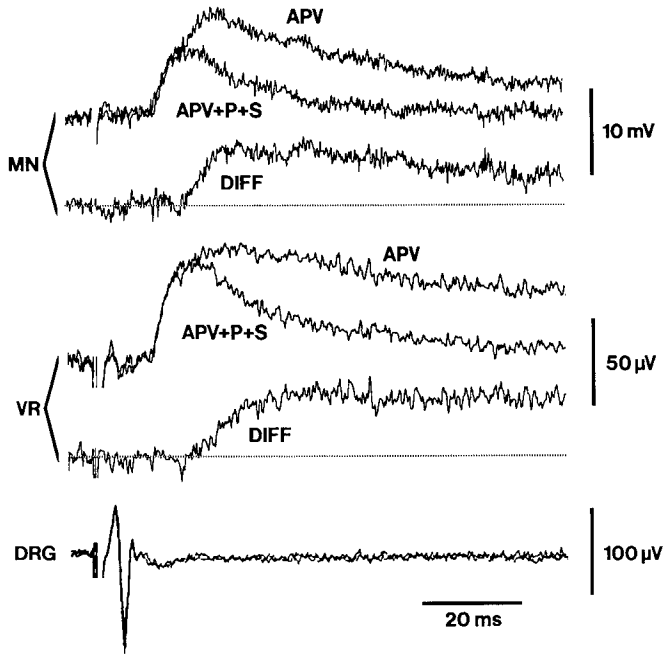

Figure 7. Effect of inhibitory blockers on the APV-resistant component of nerve-evoked motoneuron potentials. Simultaneous recordings from an LS3 motoneuron (*MN*), LS3 VR, and LS3 DRG in a stage 39 embryo. Activation of femorotibialis afferents in normal Tyrode's containing 200 μM APV produced early synaptic responses similar to those in Figure 6. Addition of 100 μM picrotoxin and 10 μM strychnine to that solution (*APV+P+S*) blocked the later portions of those responses in the motoneuron and the VR. Subtraction of the responses in APV+P+S from those in APV reveals the time course of the depolarizing IPSP (*DIFF*). The resting potential of the motoneuron was approximately −60 mV. The addition of picrotoxin and strychnine did not significantly affect the amplitude of the afferent volley in the DRG.

ulation at stage 30 was similarly incapable of evoking VR synaptic potentials in normal saline (2 of 2 embryos). In saline lacking Mg^{2+}, however, long-latency, slowly rising potentials could be produced as early as stage 28 by femorotibialis nerve stimulation (2 of 2 embryos at stage 30, 2 of 2 at stage 29, and 1 of 2 at stage 28; Fig. 10*A*). These potentials were abolished when the Mg^{2+}-free Tyrode's contained APV (2 embryos) or kynurenic acid (one embryo). Stimulation of descending tracts in the spinal cord at these stages produced VR potentials quite different in amplitude and form from those following nerve shock (Fig. 10*B*), indicating that the responses to nerve stimulation are unlikely to have resulted from passive stimulus spread to the cord. Removal of Mg^{2+} did not reveal an early component in the nerve-evoked VR potential before stage 32.

At stages 37–39 (6 embryos), both early and late components of the nerve- and DR-evoked VR synaptic potentials were completely and reversibly eliminated in solutions that block chemical synaptic transmission (0 mM Ca^{2+}, 2 mM Mn^{2+}; Fig. 11). The blockade of VR synaptic potentials in these solutions was corroborated by intracellular recording from an LS3 motoneuron in one embryo at stage 39. These observations suggest that no part of the potentials elicited in motoneurons by afferent stimulation is electrically mediated at these stages. The predominantly negative deflection caused by the afferent volley remained unchanged in the VR records, indicating that the number of afferent fibers activated by the stimulus was not significantly affected by the removal of Ca^{2+} or the addition of Mn^{2+}. In younger embryos (2 each at stages 34–35 and 32–33, 1 at stage 31, and 2 each at stages 30 and 29), most of the VR response was blocked by superfusion with 0 mM Ca^{2+}/2 mM Mn^{2+} (Fig. 11). In some of these embryos, however, the negative deflection of the afferent volley was immediately followed by a small positivity that persisted in Ca^{2+}-free, Mn^{2+}-containing saline. This latter potential may be part of the extracellular field generated by the afferent volley (Munson and Sypert, 1979). Alternatively, it may represent an electrical component to the motoneuron synaptic potential that is lost at later developmental stages.

Discussion

Development of monosynaptic connections between muscle afferents and motoneurons

Stimulation of the femorotibialis nerve in normal Tyrode's elicited a short-latency, rapidly rising potential in LS2–3 ventral roots as early as stages 32–33. Although intracellular recordings were not attempted from motoneurons at these stages, our simultaneous intracellular and ventral root recordings at stages 37–39 and previous observations (O'Donovan, 1987) demonstrate that VR potentials acccurately reflect membrane potential changes in motoneurons. VR potentials at stages 32–35 were pharmacologically similar to those at stages 37–39: the earliest component of the potential was resistant to APV and sensitive to kynurenic acid, making it similar to the Ia EPSP in mammalian motoneurons (Jahr and Yoshioka, 1986; Flatman et al., 1987) and to monosynaptic EPSPs produced in rat dorsal horn neurons by DRG cells *in vitro* (Jahr and Jessell, 1985). We therefore regard the early APV-resistant potential recorded in

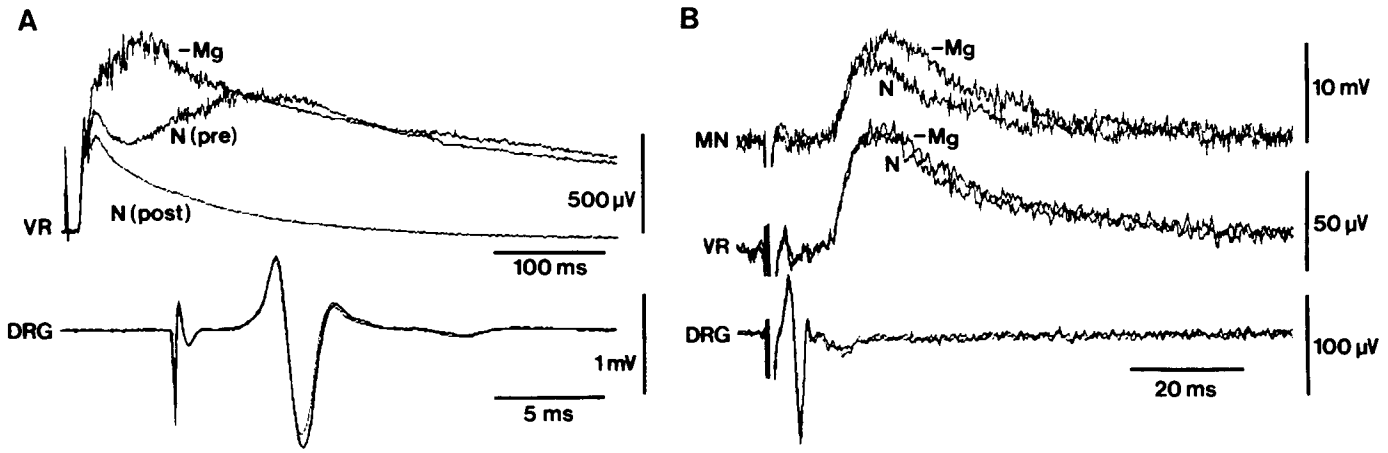

Figure 8. Effect of Mg^{2+} on nerve-evoked motoneuron potentials. *A,* Recordings from LS3 VR and LS3 DRG during stimulation of a femorotibialis nerve branch in a stage 39 embryo. The synaptic response elicited initially in normal Tyrode's, *N(pre),* was significantly enhanced after 11 min in Mg^{2+}-free Tyrode's (*−Mg*), and was markedly depressed 15 min after returning to normal Tyrode's, *N(post).* The VR response partially recovered its initial magnitude during the next 1.5 hr of continuous superfusion with normal Tyrode's. These solution changes caused only a slight change in the size of the afferent volley. *B,* Recordings from the same LS3 motoneuron, VR, and DRG as shown in Figure 7 during femorotibialis nerve stimulation. Switching the saline from normal Tyrode's containing 200 μM APV, 100 μM picrotoxin, and 10 μM strychnine (*N*) to Mg-free Tyrode's containing these agents (*−Mg*) produced a small increase in the amplitude of the monosynaptic EPSP but did not affect the afferent volley.

VRs at stages 32–33 as the first detectable monosynaptic connection between femorotibialis afferents and LS2–3 motoneurons.

Stages 32–33 should be regarded as an upper estimate of the time at which direct sensorimotor synapses form in the lumbosacral spinal cord of the chick, because only one population of afferents was studied physiologically in this investigation. However, HRP injections into the DRGs, which labeled a broader spectrum of afferents, indicated that a morphological substrate for direct connections was not present until this time. While it is possible that our techniques were not sensitive enough to reveal contacts involving very fine processes or very weak synaptic transmission, superfusion with Mg^{2+}-free saline, which demonstrated otherwise undetectable polysynaptic inputs from afferents to motoneurons at stages 28–30, did not reveal monosynaptic connections before stage 32.

The pharmacological profile of the early potential suggests that monosynaptic transmission between femorotibialis afferents and motoneurons in the chick is mediated by a glutamate-like transmitter and involves a non-NMDA type of receptor, such as the G_2 receptor identified on cultured embryonic chick motoneurons (O'Brien and Fischbach, 1986). Whether the monosynaptic potential in our preparations also included a component produced by binding to NMDA receptors was difficult to examine experimentally, since the monosynaptic EPSP could not be isolated reliably without the use of APV or high-calcium/high-magnesium solutions, both of which block these receptors.

Sensorimotor transmission was abolished at stages 37–39 by solutions that block chemical synapses. In conjunction with the report by Eide et al. (1982) that transmission is mainly chemical at stages 44–45, this finding suggests that the monosynaptic EPSP in the chick does not contain a significant electrical component during the later stages of embryonic development. In this regard, sensorimotor connectivity in the chick at these stages resembles that in mammals (Engberg and Marshall, 1979; Finkel and Redman, 1983) and differs from that in the frog (Shapovalov et al., 1978; Frank and Westerfield, 1983). Since we did not examine sensorimotor transmission with intracellular recording prior to stages 37–39, and since there was a small, positive VR potential that persisted in Ca^{2+}-free, Mn^{2+}-containing saline in some young embryos, it is not clear whether the afferent PSP in the chick is entirely chemical at all developmental stages.

Development of polysynaptic pathways between muscle afferents and motoneurons

Long-latency, slowly rising VR potentials could be elicited by femorotibialis nerve stimulation as early as stage 28. Although we did not observe afferent fibers penetrating the gray matter of the spinal cord until stage 29, Johnson et al. (1986) found a few small sensory axons within the dorsal horn at stage 28. Under our laboratory conditions, embryos reached stage 28 after 6–7 d of incubation, the same age at which Windle and Orr (1934) observed the first sensory collaterals within the spinal gray matter and the first local wing reflexes. It is conceivable that the potentials we recorded before stages 32–33 were immature versions of the monosynaptic EPSP, since similar potentials can be recorded in muscle fibers during the earliest stages in the development of neuromuscular transmission (Dennis et al., 1981). This possibility seems unlikely, however, because the potentials recorded prior to stage 32 in our preparations were completely eliminated by APV, whereas the monosynaptic potential recorded after stage 32 was largely insensitive to APV. Moreover, overlap between afferent collaterals and motoneuron dendrites was not demonstrable anatomically prior to stage 32.

Our results indicate that polysynaptic connections between femorotibialis afferents and motoneurons in the chick form earlier in development than do monosynaptic connections. Comparable results have been obtained for other afferent populations in the rat (Saito, 1979) and the frog (Forehand and Farel, 1982; Frank and Westerfield, 1983), suggesting that this is a general feature of vertebrate spinal development.

The sensitivity of these early-developing polysynaptic potentials to Ca^{2+}-free saline and to antagonists of excitatory amino acids indicates that chemical synapses exist at one or more sites in the pathway that produces them. Such potentials could be initiated by synaptic contacts between afferents and interneu-

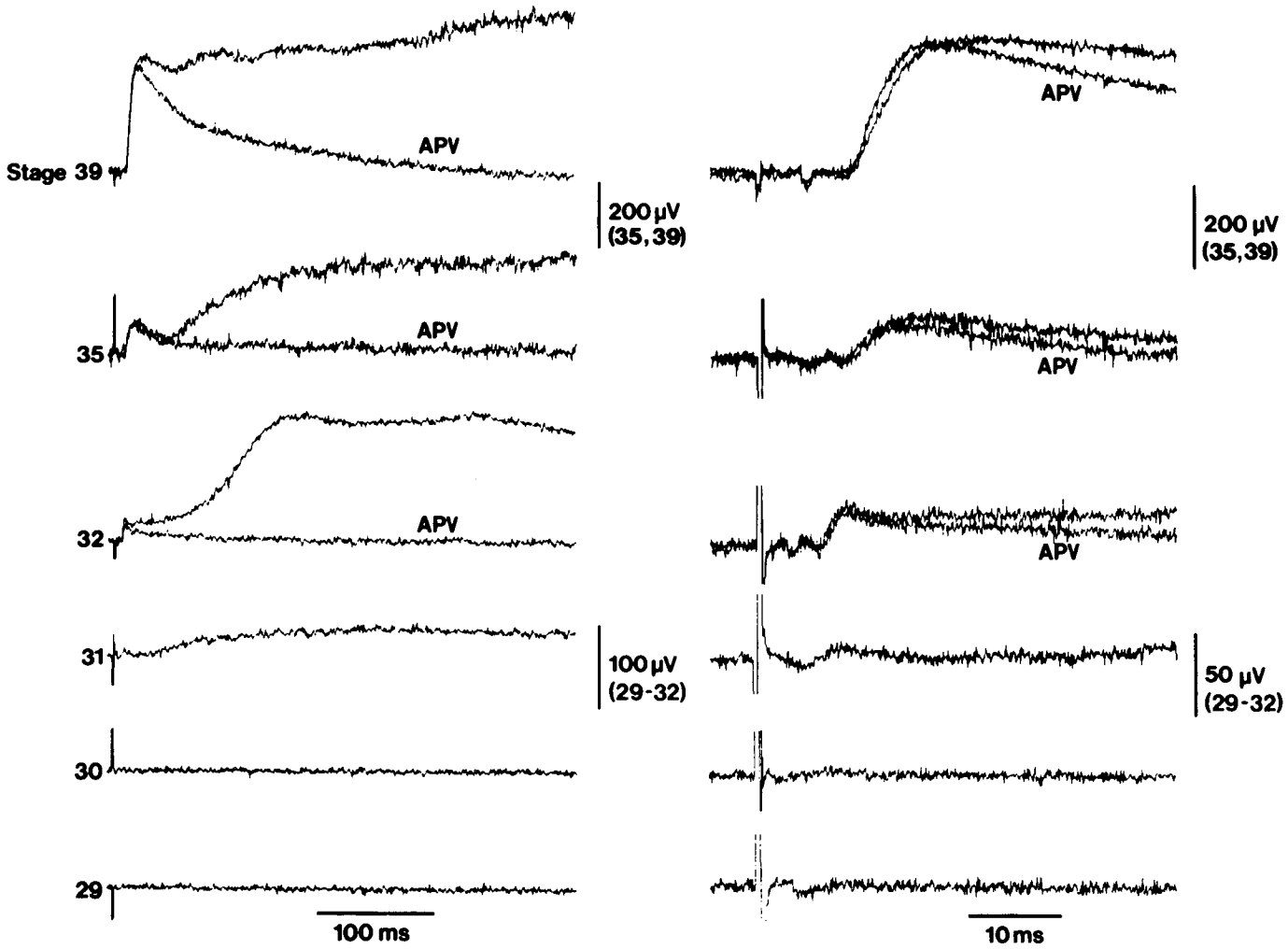

Figure 9. Development of muscle nerve-evoked VR potentials. Recordings from LS3 VR at various stages during stimulation of femorotibialis nerve branches. The initial portion of each trace on the *left* is expanded at *right*. The early component of the VR potential was clearly recognizable by stage 32. Responses shown were obtained in normal Tyrode's and, for stages 32, 35, and 39, in normal Tyrode's containing 200 μM APV. Stage 39 records are the same as those used in Figure 6. The traces from stages 35 and 39 are single responses, while those from stages 29–32 are averaged responses to 20 shocks delivered at 1/min. At stages 29–31, the stimulus intensity was adjusted to give the largest afferent volley in LS3 DR.

rons. Alternatively, the first step in this pathway might involve a nonsynaptic mechanism, such as ephaptic interactions or local depolarizations resulting from the release of K^+ ions by synchronously activated sensory fibers (Nicoll, 1979).

Because polysynaptic connections were blocked by 1 mM Mg^{2+} before stage 31 in isolated spinal cord preparations, it is not clear whether these early connections are functional *in ovo*. In more mature embryos, superfusion with Mg^{2+}-free saline caused an augmentation of sensory-evoked motoneuron potentials. Most of this augmentation appeared to have been effected via polysynaptic pathways, perhaps as a result of reductions in interneuron spike thresholds caused by the lower divalent cation concentration (Frankenhaeuser and Hodgkin, 1957). Furthermore, interneuronal transmission may have been enhanced by elimination of the voltage-dependent block of NMDA-activated ion channels by Mg^{2+} (Mayer et al., 1984; Nowak et al., 1984). The monosynaptic EPSP isolated by APV, picrotoxin, and strychnine was also slightly smaller in normal Tyrode's than in Mg^{2+}-free saline. Since the size of the afferent volley remained the same in both solutions, the depression of the EPSP by Mg^{2+} in the presence of APV may have been the consequence of a presynaptic effect of Mg^{2+} on transmitter release (Kuno and Takahashi, 1986).

Superfusion of the isolated cord with picrotoxin and strychnine at stage 33 had no effect on the APV-resistant component of the VR potential evoked by femorotibialis nerve stimulation. By stage 39, addition of these agents reduced the amplitude of this component by eliminating a depolarizing potential that began several milliseconds after the monosynaptic EPSP. Obata et al. (1978) have reported that GABA and glycine excited cultured spinal neurons from 6 and 8 d chick embryos; when applied to neurons from 10 d embryos (approximate stages 34–35), these transmitters produced inhibition. Therefore, we assume that the potential blocked by picrotoxin and strychnine at stage 39 was inhibitory. The latency of the potential suggests that it was produced polysynaptically, but the pathway involved in its production has not been determined. One candidate is recurrent (Renshaw) inhibition initiated by femorotibialis motoneurons in neighboring segments. Since we generally did not sever VRs adjacent to the root from which recordings were made, these motoneurons may have been activated antidromically by stimulation of femorotibialis nerves.

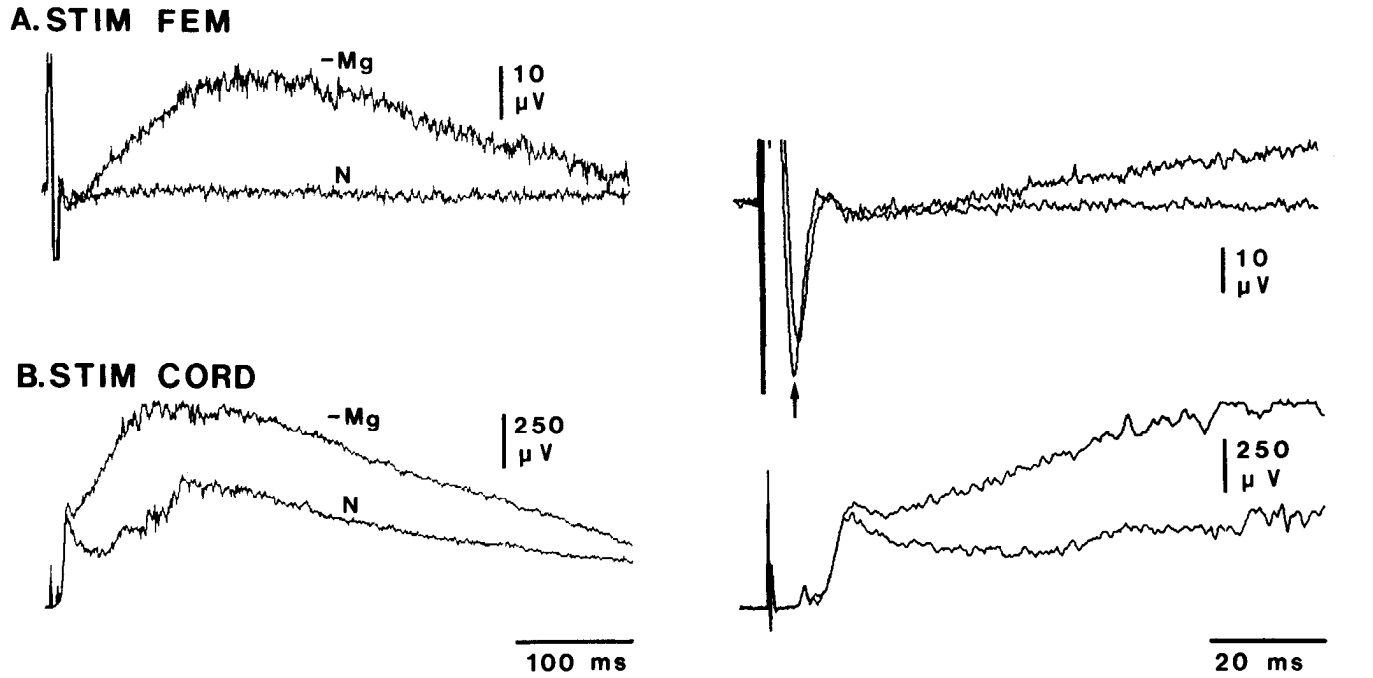

Figure 10. Effect of Mg^{2+} on nerve- and cord-evoked VR potentials in a stage 28 embryo. Recordings from LS3 VR during stimulation of a femorotibialis nerve branch (*A*) or the central spinal cord at thoracic segment 6 (*B*). The initial portions of traces on the *left* are expanded at *right*. Each trace in *A* is an average of 10 or 20 responses; traces in *B* are single events. *A*, Muscle nerve stimulation produced no response in the VR in normal Tyrode's (*N*), but elicited a long-latency potential in the absence of Mg^{2+} (*−Mg*); this solution change only slightly increased the amplitude of the afferent volley detected in the VR electrode (*arrow*). *B*, Cord-evoked potentials were reduced in size but not abolished in the presence of Mg^{2+}.

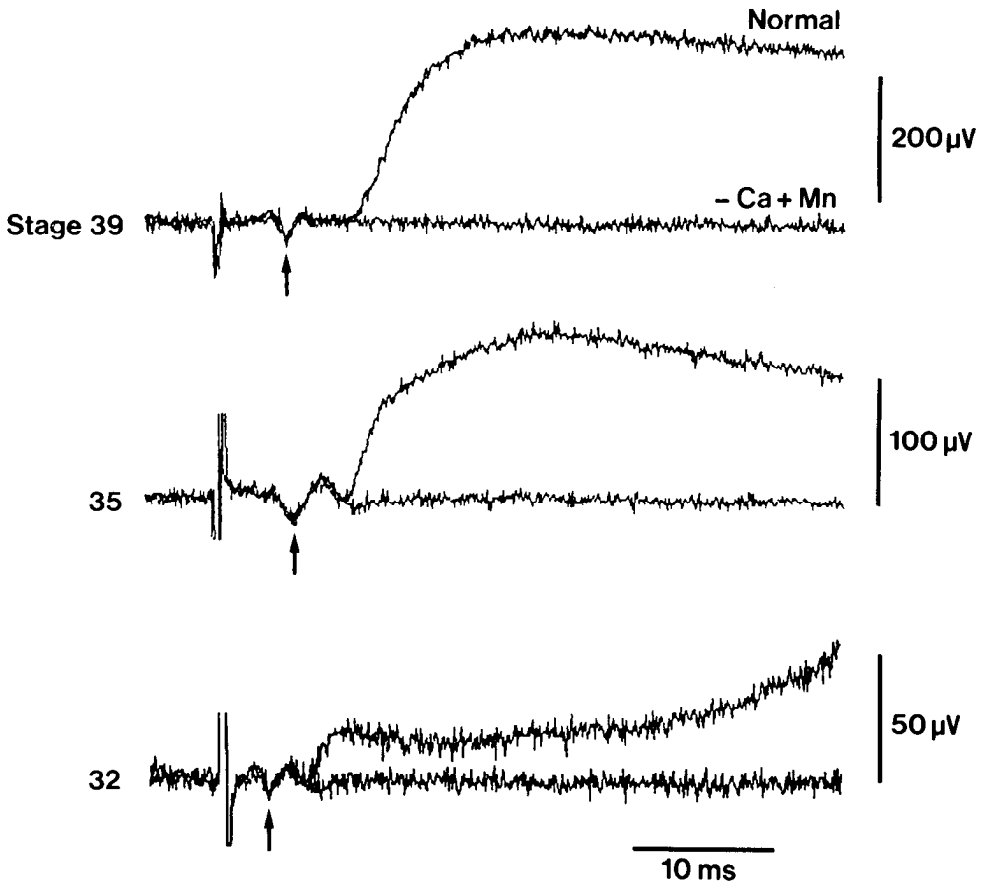

Figure 11. Chemical nature of nerve-evoked VR potentials. Recordings from LS3 VR during stimulation of a femorotibialis nerve branch in 3 embryos, stages 39, 35, and 32. At each stage, the response to nerve stimulation was recorded in normal Tyrode's and in Ca^{2+}-free Tyrode's containing 2 mM Mn^{2+}. The negative deflections caused by the afferent volleys are marked by *arrows*. Traces at stage 32 are averaged responses to 20 shocks given at 1/min.

Both depolarizing and hyperpolarizing IPSPs were recorded in motoneurons during stimulation of femorotibialis nerve branches. It is conceivable that the responses to different inhibitory transmitters are of opposite sign, or that individual motoneurons respond in opposite ways to the same transmitter. In addition, IPSPs in some motoneurons may have been reversed as a result of chloride entry or membrane depolarization caused by impalement of the cell (Velumian, 1984).

Mechanisms controlling the formation of connections between afferents and motoneurons

The outgrowth of sensory fibers into the chick hindlimb begins at stage 27 and is segmentally correct from the outset (Honig, 1982). The first monosynaptic connections between motoneurons and the central processes of femorotibialis afferents did not become detectable in our preparations until several stages later. These observations raise the possibility that the formation of synapses between muscle afferents and motoneurons might depend in some manner on a signal from the periphery, possibly from muscle. Such a mechanism has been proposed to explain the formation of appropriate connections between foreign muscle afferents and motoneurons in the bullfrog, following deletion of the ganglion that normally innervates the forelimb (Frank and Westerfield, 1982b). Myotypic specification has not been implicated in the formation of central connections of chick motoneurons, and has been disproved as a factor in the determination of connectivity of motor networks (Narayanan and Hamburger, 1971; Landmesser and O'Donovan, 1984). Nevertheless, it will be important to establish whether or not patterns of afferent connectivity are regulated by peripheral factors. This could be assessed in the chick by the use of limb rotations or shifts to produce foreign innervation of muscles (see Landmesser and O'Donovan, 1984).

The development of sensorimotor connections in the chick begins before the completion of cell death both in motoneurons (Hamburger, 1975) and afferents (Hamburger and Levi-Montalcini, 1949). It is therefore possible that cell death may be involved in the formation of these connections. In the bullfrog, the temporal relationship between these events is not as clear. Using intracellular recording, Frank and Westerfield (1983) found that afferent synapses onto brachial motoneurons formed by stage XVII, but data on cell death in this region of the cord are lacking. In the lumbar cord, sensory axons can be found in the intermediate regions of the gray matter at stages VIII–IX, and DR stimulation can elicit short-latency, apparently monosynaptic VR potentials by stage X (Forehand and Farel, 1982). Motoneuron cell death in this region occurs predominantly between stages V and X, with a large loss at stages VII–X (Farel, 1987). Hence, the establishment of sensorimotor contacts may overlap to some extent with motoneuron cell death in this species as well.

The survival of afferent neurons could depend, in part, on the formation of synaptic connections with appropriate central and peripheral targets. Davies et al. (1986) have presented evidence that the survival of cultured muscle afferents from the chick trigeminal nucleus depends on a factor derived from central neurons (brain-derived neurotrophic factor) that is distinct from the peripheral survival factor, NGF. These authors proposed that only those neurons making appropriate central and peripheral connections would receive sufficient amounts of both factors. This hypothesis predicts that the initial pattern of connectivity between afferent fibers and their central targets would include inappropriate connections. Moreover, saving afferent neurons by chronic NGF administration (Hamburger et al., 1981) should result in the maintenance of at least some of the inappropriate connections.

In chick embryos near the time of hatching (stages 44–45), lateral gastrocnemius motoneurons receive monosynaptic excitatory input from homonymous and heteronymous muscle afferents and inhibitory input from antagonistic afferents (Eide et al., 1982). This pattern of connectivity is similar to that documented in the cat (Eccles et al., 1957) and the rat (Jahr and Yoshioka, 1986). Although we observed occasional responses of opposite polarity in motoneurons during stimulation of antagonistic muscle nerves at stage 39, our data do not address the question of whether the *initial* contacts between muscle afferents and motoneurons are appropriate in the chick. This question can be answered only by examining the pattern of sensorimotor connections at the stages when these connections are beginning to form.

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