Peripheral Target Specification of Synaptic Connectivity of Muscle Spindle Sensory Neurons with Spinal Motoneurons

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The source of environmental cues determining the central connections of muscle sensory neurons was investigated by manipulating chick embryos so that sensory neurons supplied a duplicate set of dorsal thigh muscles. These neurons projected out ventral nerve pathways and along motor axons that normally project to ventral muscles but their ultimate target tissue was the duplicate set of dorsal muscles. The central connections of these sensory neurons to motoneurons supplying normal dorsal muscles were then determined with intracellular recordings in isolated spinal cord preparations. Sensory neurons supplying individual duplicate dorsal muscles made the same connections as those supplying the corresponding normal dorsal muscles; the pattern of these connections was different than that made by afferents supplying ventral muscles. Sensory neurons thus made synaptic connections appropriate for their target muscle rather than for their more proximal ventral environment. These findings suggest that the target muscle is the source of cues that determine the central connections of the sensory neurons projecting to it. Motoneurons forced to innervate novel muscle received many of the same sensory inputs they would normally receive, suggesting that motoneurons are less influenced by their target tissue than sensory neurons.

[Key words: specification, spinal cord, muscle afferent, motoneuron, synapse development, synaptogenesis, synaptic specificity]

Understanding how neurons are specified to recognize their appropriate synaptic targets is a major area of interest in neurobiology. While the neuron's ability for target recognition may result in part from information acquired prior to process outgrowth, environmental influences encountered after outgrowth begins also play an important role. One system well suited to study how environmental influences affect target recognition is the set of synaptic connections between muscle spindle sensory neurons and spinal motoneurons, the stretch reflex. Muscle afferents make monosynaptic connections only with specific subsets of motoneurons in the spinal cord. The influence of target

muscles on the development of these central connections can be studied by redirecting the peripheral axons of sensory neurons to supply novel muscles.

In frogs, the peripheral environment of developing sensory neurons plays a major role in directing the selection of their targets in the spinal cord (Frank and Westerfield, 1982a,b; Smith and Frank, 1987). When thoracic sensory axons were redirected to project along the brachial nerve into the developing front leg of a tadpole, they supplied muscle spindles in various forelimb muscles. Although these neurons normally do not make direct connections to motoneurons, they responded to their novel peripheral environment by forming synaptic connections with the now appropriate forelimb motoneurons. Some cue within the brachial nerve pathway, the forelimb motor axons, or the target muscles themselves resulted in the formation of novel, but now appropriate central connections. This cue appears to be molecular, as blockade of neuronal activity during the period of synaptogenesis does not interfere with the development of the normal pattern of connections (Frank, 1990; Mendelson and Frank, 1991).

To determine whether the nerve pathway, the motor axons, or the target muscle is the source of this cue, we arranged that sensory afferents would project to novel muscles but would reach these novel targets via their *normal* pathways and in association with their *normal* motor axon partners. In chicken embryos, the ventral half hindlimb bud was surgically replaced with a dorsal half from a donor embryo before any axons had grown into the limb. These embryos develop with a duplicate set of dorsal muscles in the ventral half of the limb, which are supplied by motoneurons normally innervating ventral muscles (Lance-Jones, 1986). Sensory axons thus project through proximal ventral muscle nerve pathways and in association with motoneurons that normally supply ventral muscle, but peripherally these afferents encounter a duplicate set of dorsal muscles. The central connections of these afferents were then assessed by making intracellular recordings from motoneurons supplying the normal (host) set of dorsal muscles.

The pattern of connections established was appropriate for the duplicate dorsal muscle supplied but inappropriate for the associated motoneurons or proximal pathway, suggesting that the molecular information determining these connections resides primarily in the target tissue itself. Motoneurons were less influenced by their target muscle as demonstrated in a separate manipulation.

Short reports of some of these results have appeared previously (Wenner and Frank, 1990; Wenner et al., 1991; Frank and Wenner, 1993).

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Figure 1. Morphology of thigh muscles in normal chick embryos and embryos with a duplicate set of dorsal muscles. Photomicrographs are shown of cross sections through the mid-thigh of E10 control (*A*) and experimental (*B*) embryos. Similar views are given in E14 control (*C*) and experimental (*D*) embryos. By E14, some duplicate dorsal muscles are highly atrophied, especially the duplicate FTmed and FTtext (as well as anterior iliotibialis and ambiens, which are not clearly apparent in *D*). Despite this atrophy, individual duplicate muscles are readily identified by their position in the limb. Dorsal muscles include the FTint (1), ambiens (2), sartorius (3), anterior iliotibialis (4), FTmed (5), FTtext (6), posterior iliotibialis (7), and the iliofibularis (8). Ventral muscles include the caudioflexorius (9), ischioflexorius (10), and superficial (11) and deep (12) adductors. Duplicate muscles are labeled with an asterisk. Scale bars, 0.5 mm.

Materials and Methods

Surgery. White Leghorn chicken embryos were incubated at 37°C in a forced draft incubator and staged according to Hamburger and Hamilton (1951). Duplicate sets of dorsal musculature were created by replacing the ventral half of the hindlimb bud from a host embryo with a dorsal half limb bud from a comparably staged donor as described by Lance-Jones (1986). Most manipulations were performed on stage 18 embryos (range, 17–19), before motor or sensory axons have entered the limb (Hollyday, 1983; Tosney and Landmesser, 1985). Embryos with reversed sets of calf musculature were created by rotating the limb bud at stage 18, as described previously (Whitelaw and Hollyday, 1983).

Identification of muscles in experimental embryos. Hindlimbs with dorsal duplications contained a set of normal and corresponding duplicate dorsal thigh muscles innervated by the ischiadic nerve trunk (normal and duplicate posterior iliotibialis and iliofibularis muscles, and the duplicate femorotibialis external muscle) and crural nerve trunk. The present study examines only the crural nerve trunk and associated muscles because dissection of the ischiadic nerve trunk was technically more difficult. The dorsal branch of the crural nerve trunk innervated the femorotibialis external (FTtext), femorotibialis medial (FTmed), femorotibialis internal (FTint), anterior iliotibialis, ambiens, and sartorius muscles of the thigh. The ventral branch of the crural nerve trunk innervated the corresponding duplicate set of dorsal muscles in the thigh (except the duplicate FTtext; see above) instead of its normal ventral muscle targets (obturator, superficial and deep adductors). This trunk had a normal branching pattern proximal to the duplicated tissue. At stage 36 (E10), both the normal dorsal (host) and duplicate dorsal (donor) thigh muscles were similar to those in normal embryos (Fig. 1*A,B*). Occasionally, however, the set of duplicate muscles was not complete; the FTint, for example, was sometimes fused to the corresponding normal muscle or was completely absent.

By stage 40 (E14), when recordings were made from motoneurons, duplicate muscles were atrophied, surrounding only one-fourth of the femur. This is approximately the size of the ventral muscles in normal embryos (Fig. 1*C,D*). The ventral crural nerve trunk supplying these muscles was also thinner than normal. Atrophy was especially apparent in the duplicate FTmed and FTtext muscles (Fig. 1*D*). Despite this at-

rophy the basic pattern of the duplicated muscles was clear enough to permit their unambiguous identification and also, therefore, the identification of the nerves that supplied them.

In embryos with dorsoventrally rotated limbs, the musculature was approximately normal except for the 180° rotation distal to the knee. Muscle nerve patterns were normal except that the dorsal muscle nerves originated from the ventral ischiadic nerve trunk (located more posteriorly) and vice versa (see Whitelaw and Hollyday, 1983, for a more complete description). Rotated muscles were somewhat atrophied by stage 40, although sham surgeries (limb bud removed but not rotated before reattachment) did not result in atrophy.

Histology. Cell bodies of motor and sensory neurons were retrogradely labeled with HRP applied to the sartorius muscle nerve or the ventral crural nerve trunk. Whole spinal cords were processed for visualization of HRP using a cobalt intensification technique (Landmesser, 1978; Frank and Sah, 1986). Spinal cords were placed in fixative (2% glutaraldehyde containing 0.1 M phosphate buffer, pH 7.2) for 2 hr, washed 2 × 1 hr in Tris buffer, 2 hr in 0.1 M NaBH₄ in Tris, and again 1 hr in Tris, after which the cord was preincubated for 2 hr in a glucose oxidase-diaminobenzidine reaction solution (Frank and Sah, 1986) with one solution change. The DAB reaction, initiated by addition of β-D-glucose (Sigma), was run for 2.5 hr at room temperature with agitation, in the dark.

The spinal cord was embedded in paraffin, serially sectioned transversely at 10 μm, and stained with cresyl violet. Paraffin cross sections (10 μm thick) of control and duplicate dorsal thigh muscles at stage 36 and 40 were stained with cresyl violet or hematoxylin and eosin (Lance-Jones, 1988).

Distribution of motoneurons projecting to relocated muscles. To confirm that our manipulations caused medially positioned motoneurons (MP) to supply duplicated dorsal muscles, as originally shown by Lance-Jones (1986), we plotted the position of motoneurons projecting via the ventral crural nerve trunk versus those projecting to the sartorius muscle (a dorsal thigh muscle and hence with laterally positioned somata—LP-sartorius) on tracings of transverse sections of the spinal cord in 5 control embryos using the medial and lateral borders of the lateral motor column (LMC) for reference. We then categorized labeled mo-

motoneurons in four experimental and two control embryos as MP or LP-sartorius based on these normal maps. In the control embryos, 95.5% (range, 94–97) of the motoneurons projecting in the ventral crural nerve trunk were classified as MP; 95.3% (range, 94–97; $n = 3$) of the sartorius motoneurons were classified as LP-sartorius. Motoneurons projecting via the ventral crural nerve trunk in embryos with duplicate dorsal thigh muscles were also classified as MP (95%; range, 94–96; $n = 2$) despite the fact that these motoneurons innervated the duplicate dorsal muscles. The normal sartorius muscle in these embryos was innervated by its normal complement of motoneurons; 95.5% (range, 93–98; $n = 2$) of motoneurons projecting to the normal sartorius muscle were classified as LP-sartorius. Analogous results were obtained for motoneurons projecting to rotated calf muscles, as originally reported by Whitelaw and Hollyday (1983). Dorsal calf muscles were supplied by motoneurons located medially in the LMC while motoneurons supplying ventral calf muscles were located laterally. These results with rotated calf musculature, as well as a complete account of the motoneurons projecting to duplicate dorsal thigh muscles, has been published previously (Wenner, 1993). Motoneurons within the proximal nerve pathways of both types of experimental embryos were therefore the normal ones for those pathways, implying that the duplicated or rotated muscles received inappropriate motor innervation.

Electrical recordings from motoneurons. The pattern of synaptic connections between muscle afferents and motoneurons was determined by making intracellular recordings from motoneurons using an isolated spinal cord preparation (stage 39–40; Lee et al., 1988). Eggs were cooled for 10–20 min in ice water, and the embryos were dissected in 12–15°C recirculating Tyrode's solution that warmed slowly to room temperature during the dissection. Other details are described in Mendelson and Frank (1991). Data were collected only from cells with an antidromic action potential and a minimum resting potential of -40 mV. A further criterion was that each motoneuron received an input larger than 1 mV from at least one muscle nerve. Similarly, a muscle nerve was included in the analysis of an embryo only if it evoked a synaptic potential of 1 mV or more in at least one motoneuron.

Individual traces (usually 5–20) were stored digitally and averaged on line using a stimulus frequency of ~ 1 Hz. Monosynaptic components of the average potentials were measured using model synaptic potentials recorded in each preparation (Mendelson and Frank, 1991). The synaptic latencies of input from afferents supplying normal muscles in experimental embryos were indistinguishable from those in normal embryos, and were used as models for analyzing inputs from duplicate dorsal muscles. Latency profiles for stimulation of each muscle nerve were generated in each preparation. The earliest potentials were considered to represent monosynaptic potentials; monosynaptic latencies generated in this way were always shorter than IPSPs (which are polysynaptic) and corresponded well with other reports in this preparation (see Lee et al., 1988; Lee and Donovan, 1991; Mendelson and Frank, 1991).

Two methods were used to circumvent problems associated with the measurement of homonymous synaptic inputs. The nerve stimulus was reduced until it was just subthreshold for antidromic activation of the motoneuron (Mendelson and Frank, 1991). If a monosynaptic input was still visible, its amplitude was measured. This method usually results in an underestimate of the synaptic input because not all sensory afferents are stimulated. The motoneuron was stimulated orthogradely by current injection through the pipette while the muscle nerve was stimulated peripherally. If the two stimuli were correctly timed, the resulting collision of action potentials in the motor axon revealed the underlying sensory synaptic potential. If the collision technique was unsuccessful and the monosynaptic potential produced by the first method was less than 1 mV, the data were not used in subsequent analyses.

Criteria for inclusion of experimental results in analysis of synaptic patterns. The variability of muscle and nerve patterns in individual experimental embryos combined with the difficulty of making reliable recordings in these embryos required the adoption of a rigorous set of criteria for accepting data from each embryo. The anatomical pattern of duplicate muscles had to be clearly discernible and motoneurons innervating those muscles had to be located in a specific region of the spinal cord (i.e., duplicate dorsal muscles innervated by motoneurons positioned medially in the spinal cord; see first section of Results). Further, at least five motoneurons yielding sufficient data to make a reliable estimate of monosynaptic latencies had to be obtained in each embryo. The electrophysiological data reported here were obtained from 23 embryos with duplicate dorsal muscle, 5 embryos with rotated limbs, and 31 control embryos.

Results

Identity of motoneurons supplying novel muscles

The strategy of these experiments was to create a situation where sensory neurons projected to muscles in association with motor axons that would not normally innervate those muscles. In agreement with earlier studies (Whitelaw and Hollyday, 1983; Lance-Jones, 1986), we found the motor innervation of the novel muscles (the duplicate set of dorsal thigh muscles or the rotated set of calf muscles) was inappropriate; that is, medially positioned motoneurons (MP) that normally project to ventral muscles projected to dorsal muscles (see Materials and Methods). Motor axons projecting via a particular proximal nerve trunk were the ones that would normally project in that trunk but were inappropriate for the muscle they supplied.

Distal versus proximal influence on sensory neurons

A simple test to determine the source of influence (peripheral or proximal) on muscle sensory neurons was performed by stimulating the ventral crural nerve trunk innervating the duplicate dorsal thigh musculature. Recordings were obtained from four different types of laterally positioned (LP) motoneurons that innervated host dorsal muscles (sartorius and the FTmed, FTint, and FTint), as illustrated schematically in Figure 2. The monosynaptic responses recorded in normal sartorius or FTint motoneurons were different in experimental and control embryos. For the case shown in Figure 3, the input to a sartorius motoneuron was considerably larger in the experimental embryo. The *average* input to all normal sartorius motoneurons was significantly larger in the experimental embryos, as was the case for inputs to normal FTint motoneurons (Fig. 4). The change in input to normal LP motoneurons was selective, in that some inputs were increased (to sartorius and FTint motoneurons) while others were not changed (to FTmed or FTint motoneurons). Therefore the novel target muscles, rather than the proximal environment, of these sensory neurons selectively influenced the functional connections they made with normal motoneurons.

Peripherally determined synaptic connectivity of sensory neurons

To determine if these changes in connectivity were functionally appropriate for the individual muscle supplied, we carried out a second series of experiments in which the individual branches of the ventral crural nerve trunk supplying the various duplicate muscles were stimulated individually. In this way, the inputs from afferents supplying individual normal and duplicate muscles could be compared in four different types of normal LP motoneurons (sartorius, FTint, FTmed, and FTint). For each motoneuron, synaptic potentials were recorded in response to stimulation of the nerve supplying the duplicate and normal sartorius and FTint muscle.

Examples of these synaptic inputs to two different motoneurons are illustrated in Figure 5. Stimulation of either the duplicate or host (normal) sartorius muscle nerve produced large monosynaptic potentials in a normal sartorius motoneuron. In contrast, sensory afferents in either the duplicate or host FTint muscle nerve provided very little input to this motoneuron (Fig. 5A). FTint motoneurons, on the other hand, received large inputs from duplicate and normal FTint muscle nerves, but inputs from duplicate and normal sartorius muscle nerves were weak or nonexistent (Fig. 5B). For both motoneurons, strong inputs were observed from "homonymous" sensory neurons, just as in normal embryos ("homonymous," meaning neurons projecting to

Figure 2. Diagram of experimental design. A muscle sensory neuron projects with MP motoneurons (which would normally project to ventral muscles) along proximal ventral nerve pathways but peripherally supplies a duplicate dorsal muscle. Dashed line indicates the plane separating proximal host and distal donor tissue. The central connections of these muscle sensory neurons were assessed by intracellular recordings from LP motoneurons projecting to their normal dorsal muscle targets. The patterns of these central connections onto the different types of LP motoneurons were then determined to be appropriate for target muscle (i.e., similar to those of normal dorsal muscle afferents) or muscle nerve pathway and MP motoneurons (i.e., similar to those of ventral muscle afferents).

the same muscle type, whether duplicate or normal). Conversely, inputs that were small from host dorsal muscle nerves were also small from the corresponding duplicate muscle nerves.

To check that the manipulation creating the duplicate dorsal muscles did not influence the sensory or motoneurons supplying host dorsal muscles, we compared the pattern of connections between these neurons to the corresponding pattern in normal embryos. This comparison is shown as a scatterplot in Figure 6A, where each point represents the average monosynaptic input a particular type of motoneuron received from a particular type

of muscle afferent (4 types of motoneurons $\times$ 2 types of muscle afferents = 8 points). Inputs to LP motoneurons in normal embryos are shown on the x-axis, while the corresponding inputs from host dorsal muscle nerves to LP motoneurons in experimental embryos are indicated on the y-axis. The high degree of correlation between inputs in normal and experimental embryos ($r = 0.88$, slope = 1.01) suggests that the connections between sensory and motor neurons supplying host dorsal muscles were not noticeably affected by the surgery or the presence of the duplicate set of muscles.

Figure 3. Intracellular recordings of synaptic input to normal sartorius motoneurons from sensory axons in the ventral crural nerve trunk of experimental and control embryos. Both synaptic potentials include monosynaptic components (note similar short latencies, indicated by arrows), but the sensory afferents supplying duplicate dorsal muscles (Experimental) evoked a larger potential than those supplying normal ventral thigh muscles (Control). Calibration pulse: 2 msec, 0.5 mV.

Figure 4. Comparisons of average monosynaptic input to four types of normal LP motoneurons from sensory axons in the ventral crural nerve trunk in experimental (shaded bars) and control (open bars) embryos. FText and sartorius motoneurons receive stronger sensory input in experimental embryos. Error bars indicate 1 SE; (n) indicates number of sampled motoneurons. Statistical significance was determined using two-tailed t test: *, $p < 0.05$; **, $p < 0.005$.

Figure 5. Comparison of synaptic potentials evoked by sensory afferents supplying duplicate dorsal and corresponding host dorsal muscles in normal LP motoneurons. The text *below* each trace indicates the muscle nerve that evoked the response. In *A*, afferents supplying duplicate and host sartorius muscles make strong monosynaptic projections with a sartorius motoneuron while the input from afferents supplying duplicate and host FTint muscles was small. This pattern was reversed in an FTint motoneuron in the same embryo (*B*), so in both cases the sensory input from the duplicate muscle was normal, that is, appropriate for that muscle. Arrows indicate the onset of polysynaptic potentials. Calibration pulse: 2 msec, 0.5 mV.

A similar comparison could then be made between the inputs from sensory afferents supplying duplicate dorsal muscles to LP motoneurons and the corresponding connections between sensory and motor neurons supplying dorsal muscles in normal embryos. This comparison is shown as a scatterplot in Figure 6*B*, analogous to that in Figure 6*A*, where the *x* and *y* values of each point indicate the average input from afferents supplying normal and duplicate dorsal muscles, respectively. Just as in the specific examples shown in Figure 5, inputs that were large in normal embryos were the same ones that were large from the corresponding duplicate muscles in experimental embryos, including the two sets of “homonymous” connections. Each group of afferents supplying duplicate dorsal muscles (sartorius and FTint) made a distinct and essentially normal pattern of connections centrally, strong to some motoneurons and weak to others. The excellent correlation between inputs from normal and duplicate muscles ($r = 0.91$) argues that afferents projecting to a novel muscle made central connections appropriate for the muscle they supplied. This is strong evidence supporting the idea that the

target tissue determines the central connections of muscle afferents.

Although the *pattern* of inputs from afferents supplying duplicate muscles was appropriate for these novel targets, the *amplitudes* of these inputs were smaller than normal, about two-thirds their normal size (slope of least squares fit to data in Fig. 6*B* is 0.63). Another difference between the inputs from afferents supplying duplicate versus corresponding normal muscles is that no synaptic potentials were recorded in response to stimulation of the duplicate FTmed muscle nerve in any of the experimental embryos. Sensory neurons did project to this muscle in at least one embryo, as indicated by dorsal root potentials recorded after stimulation of this nerve. A possible explanation of these results is considered in the Discussion.

Influence of the novel targets of motoneurons on their synaptic input from muscle afferents

To determine if the target muscle of a motoneuron influenced the sensory inputs that motoneuron receives, we examined the synaptic input from muscle afferents supplying normal dorsal muscles to MP motoneurons projecting to novel targets, the duplicate set of dorsal muscles. Unfortunately very few stable intracellular recordings were obtained from MP motoneurons in these preparations. A different surgical manipulation was therefore used to address this issue. The distal part of the limb bud was rotated so that the calf musculature developed in a dorso-ventrally reversed position (see Materials and Methods). Motoneurons normally innervating ventral calf muscles (MP motoneurons) innervated dorsal calf muscles, and LP motoneurons (normally innervating dorsal calf muscles) innervated ventral calf muscles.

A complication inherent in this manipulation is that all motor and sensory neurons projected to novel targets, so changes in the central connections may have resulted from effects on motoneurons, sensory neurons, or both. Results from the first set of experiments, however, showed that sensory neurons make central connections appropriate for their target muscles. To interpret the results from the present experiments, we therefore assumed that sensory neurons supplying a particular calf muscle made central projections appropriate for that muscle.

In normal embryos, LP motoneurons, which innervate dorsal calf muscles, receive strong input from sensory afferents supplying dorsal calf muscles, while MP motoneurons, innervating ventral calf muscles, receive comparably little input from these afferents (left and right columns of Fig. 7, respectively). If LP motoneurons forced to innervate ventral calf muscles had been specified by their novel peripheral targets to become MP motoneurons, they should not have received inputs from dorsal muscle afferents. In fact, however, afferents supplying the dorsal calf muscles did provide significant input to these rerouted LP motoneurons (middle column of Fig. 7). These results suggest that the novel target muscles of motoneurons do not completely dictate the sensory inputs these motoneurons receive. This conclusion is overly simplistic however. Motoneurons innervating muscles in these rotated limbs often received additional inputs that were not consistent with their spinal cord position or the muscle they supplied (Wenner, 1993). It was not possible to determine more precisely the influence of the novel target on a motoneuron's sensory inputs, however, because the particular calf muscle each rerouted motoneuron was originally specified to innervate was unknown.

Figure 6. Comparison of afferent input in experimental and control embryos. **A**, The scatterplot compares the functional connectivity between sensory and motor neurons supplying dorsal muscles in control and operated limbs. *Y* values indicate the average monosynaptic input from afferents supplying either the host sartorius (open symbols) or the host FTint (solid symbols) muscle onto four types of normal LP motoneurons (FTint, FTmed, FText, and sartorius). Corresponding *x* values indicate the same sensory-motor connections in control embryos. The

Figure 7. Muscle sensory input to calf motoneurons innervating normal and novel calf muscles. The novel motor projections were produced by making embryos with dorsoventral limb rotations. In control embryos (left and right bars), LP motoneurons innervating dorsal calf muscles receive strong synaptic input from sensory afferents supplying dorsal muscles (left bar) whereas MP motoneurons, which innervate ventral calf muscles, receive little input from these same afferents (right bar). In embryos with dorsoventral rotation of the calf, LP motoneurons now innervating ventral calf muscles receive significantly more input from dorsal muscle afferents than the motoneurons normally innervating ventral calf muscles (compare middle and right bars). Motoneurons innervating novel muscles still receive many of the same sensory inputs they normally would, despite the fact that these inputs are now inappropriate. Error bars indicate 1 SE; (*n*) indicates number of sampled motoneurons.

Discussion

Identity of sensory neurons supplying novel muscles

Several observations suggest that the sensory neurons projecting to duplicate dorsal muscles would have supplied ventral thigh muscles in normal embryos. Sensory and motor neurons supplying a particular muscle are each located at the same segmental level following a variety of different surgical manipulations whether they supply normal or abnormal targets (Honig et al., 1986). Similar results are obtained after neural crest transplantations which cause sensory ganglia to arise from novel segmental levels (Scott, 1986); the sensory neurons project to the same muscles as the motoneurons at that segmental level. This

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similarity between synaptic inputs in normal and manipulated limbs ($r = 0.88$, $p < 0.01$, slope of least squares fit = 1.01) shows that the manipulation did not interfere with the formation of appropriate central connections. *Y* values are from 196 sensory-motor pairs; *x* values, from 261 sensory-motor pairs. Four points located near the origin partially obscure each other. **B**, This scatterplot compares the synaptic input from sensory axons supplying duplicate (*y*-axis) versus the corresponding normal dorsal muscle (*x*-axis) in control embryos onto the four different types of LP motoneurons. The high correlation between these two sets of values ($r = 0.91$, $p < 0.005$, slope of least squares fit = 0.63) shows that afferents supplying normal and duplicate muscles made a similar pattern of connections with LP motoneurons. Error bars indicate 1 SE. *Y* values are from 92 sensory-motor pairs; *x* values, from 261 sensory-motor pairs and are the same data as in **A**. Four points located near the origin partially obscure each other.

suggested to the authors of both studies that sensory neurons may simply project along adjacent motor axons. When motoneurons are surgically removed early in development, sensory afferents fail to project to muscles (Landmesser and Honig, 1986), further supporting the idea that sensory axons are guided to muscles by associating with nearby motor axons. Finally, in our experimental embryos, the segmental distribution of sensory neurons projecting to duplicate dorsal muscles was like that of sensory neurons normally supplying ventral, but not dorsal, thigh muscles (data not shown; refer to Wenner, 1993). Sensory neurons supplying dorsal versus ventral muscles do not have characteristic locations within a single DRG, so we could not definitively determine if the neurons supplying duplicated dorsal muscles would have normally supplied ventral thigh muscles. Because sensory axons supplying the duplicate dorsal muscles projected in the ventral crural nerve trunk and in association with MP motoneurons, however, this is a likely possibility.

Influence of peripheral targets on central connections of muscle sensory neurons

These results help determine the location of the peripheral cues leading to the formation of functionally appropriate synaptic connections between muscle sensory and motor neurons. Sensory neurons forced to supply a duplicate set of dorsal limb muscles, instead of their likely normal target (ventral limb muscles), established central connections appropriate for those dorsal muscles. The muscle afferents made these connections despite the fact that they projected in the ventral crural nerve trunk and in association with motoneuronal axons that normally would have projected to ventral muscles. The cues determining these connections are therefore unlikely to come from motor axons or other structures in the proximal nerve pathways but instead reside in the target tissue itself.

This conclusion is based on two sets of observations. In the first set, the entire muscle afferent population of the ventral crural nerve trunk was stimulated together. These afferents supplied ventral limb muscles in normal embryos, while in experimental embryos they supplied the duplicate dorsal muscles (Fig. 2). The inputs these afferents formed with normal LP motoneurons (innervating their normal dorsal muscle targets) were quite different in normal and experimental embryos, arguing that the novel target muscles had influenced their pattern of central connections. The specificity of this peripheral influence was studied in a second set of experiments in which individual nerves to two of the duplicate dorsal muscles were stimulated separately. Sensory afferents in each of these nerves provided nearly the identical pattern of inputs to normal LP motoneurons that afferents supplying the corresponding normal dorsal muscles did. The implication is that an individual target muscle can specifically determine the central connections of the sensory neurons supplying it.

A tacit assumption in this study was that the anatomical position and appearance of individual duplicate dorsal muscles provided an accurate indication of their actual identity at a molecular level. Even if muscles normally provided molecular cues to specify the central connections of the sensory neurons that supply them, gene expression in duplicate dorsal muscles might have been sufficiently altered to obliterate the cues. That these central connections were highly predictable based on the individual muscle supplied implies that this assumption was probably correct. Individual duplicate muscles were identified correctly, and cues in these muscles were normal. The robustness

of these cues in manipulated embryos should prove useful in future experiments aimed at identifying the specific molecules that mediate these effects.

Potential mechanisms of peripheral influence

There are two general ways in which central connections of muscle afferents might be determined by their peripheral targets. One possibility is that the central connections of a muscle afferent are specified by its particular peripheral target. The chemical signal might arise from the muscle itself or from the surrounding connective tissue. Molecular information could be transported retrogradely to the cell body in the DRG, either directly or via some second messenger cascade, where it could regulate the expression of proteins specific for the particular muscle supplied by that sensory neuron. These proteins could then be expressed on the central arbors of the sensory afferents allowing recognition of motoneuronal dendrites with complementary labels. Distinct labels for each of the various limb muscles would be required in order to specify uniquely the central connections of the afferents that supply them. At first, this might seem to require an unrealistically large number of different labels. A provocative example of such molecular diversity, however, is provided by the recent finding that olfactory receptor neurons each express mRNA encoding a single one of several hundred different olfactory receptor proteins (Ressler et al., 1994; Vassar et al., 1994). The message, and presumably the protein, is found in the sensory axons where they arborize in the olfactory bulb, and all the axons expressing a particular mRNA project to only one or two of the hundreds of different target glomeruli in the bulb.

The other possibility is that both central and peripheral targets of sensory neurons are prespecified before axonal projections are formed. The influence of peripheral targets on central connectivity would then be mediated either by specific guidance of sensory axons to particular target muscles, as for motoneurons, or by random innervation of each muscle by many different types of prespecified sensory neurons followed by a highly specific apoptosis of all afferents projecting to incorrect muscles. Specific guidance seems unlikely for the reasons given at the beginning of the Discussion. Because duplicate dorsal muscles were innervated by inappropriate motoneurons, prespecified sensory neurons would probably have projected incorrectly as well. The second mechanism, random projections followed by selective apoptosis, would require that each muscle be supplied by sensory neurons prespecified for every muscle in the limb. The large number of excess sensory neurons that would be necessary makes the idea unattractive, but there is no direct evidence against it.

In several other systems there is direct evidence for peripheral specification of neuronal phenotype rather than selective apoptosis. Individual sympathetic neurons innervating sweat glands change their neurotransmitter phenotype from noradrenergic to cholinergic based on contact with their target (Landis and Keefe, 1983; Leblanc and Landis, 1986; Landis et al., 1988). Similarly, the central arborizations of Retzius neurons in leech ganglia are determined by the peripheral targets (body wall vs reproductive tissue) they encounter during embryonic development (Loer and Kristan, 1989a,b). Regardless of the mechanism by which peripheral cues determine central connectivity of sensory afferents in the stretch reflex system, however, the location of these cues is in or near the target muscles.

Muscle atrophy

The atrophy of duplicate dorsal muscles may explain why synaptic potentials produced by sensory afferents supplying these muscles were smaller than normal. Atrophied muscles might be capable of maintaining fewer spindle afferents, or the terminal arbors of afferents supplying these muscles might be smaller than normal. The clearest examples of peripheral specification were for duplicate muscles with less atrophy (sartorius and FTint). Afferents supplying the more atrophied duplicate FTmed muscle provided no input to any motoneuron. Atrophy of rotated muscles might also account for the smaller sensory inputs observed in this manipulation (Fig. 7, middle vs left columns).

The effects of novel peripheral targets on the connectivity of motoneurons

The effect of peripheral targets on motoneurons was examined in embryos with dorsoventrally rotated limbs. Motoneurons supplying novel muscles in these embryos, still received many of the same inputs as normal. Although the two experimental preparations are not strictly comparable this suggests that novel peripheral targets do not completely dictate a motoneuron's input from muscle afferents. This is consistent with results from earlier studies; when motoneurons were made to innervate novel muscles, their spontaneous activity patterns, of interneuronal origin, were approximately normal (Landmesser and O'Donovan, 1984; O'Brien et al., 1990). Because the specific dorsal or ventral calf muscles these motoneurons normally supplied were unknown, however, it was not possible to interpret some aspects of the pattern of sensory input that had been modified by the novel targets. Nevertheless, these results suggest to us that motoneurons may have a more limited ability than sensory neurons to react appropriately to novel peripheral targets.

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