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SPECIFICATION OF SYNAPTIC CONNECTIONS MEDIATING THE SIMPLE STRETCH REFLEX

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Summary

A variety of mechanisms underlie the specification of synaptic connections during development. In the monosynaptic stretch reflex in vertebrates, sensory neurones innervating muscle spindles are not determined until they make contact with a particular muscle. Instead, the muscle they supply appears to specify the pattern of central connections they establish with motoneurones. Developing thoracic sensory neurones made to project to novel peripheral targets in the forelimb of tadpoles project into the brachial spinal cord, something they never do in normal frogs. Moreover, these foreign sensory neurones make monosynaptic connections with the now functionally appropriate brachial motoneurones. Normal patterns of neuronal activity are not necessary for the formation of specific central connections. Neuromuscular blockade of developing chick embryos with curare during the period of synaptogenesis does not prevent the formation of correct sensory-motor connections. Competitive interactions among the afferent fibres also do not appear to be important in this process. When the number of sensory neurones projecting to the forelimb is drastically reduced during development, each afferent fibre still makes central connections of the same strength and specificity as normal. Together, these results suggest that peripheral targets induce some molecular change in developing sensory neurones such that they can recognize their appropriate synaptic partners in the spinal cord.

Introduction

One hallmark of the developing nervous system is the highly ordered sets of synaptic connections that develop among neurones. These specific connections are a product of at least several different mechanisms operating in concert. To reach a particular target area, a neurone must be born within a restricted window of time and must possess the requisite cellular machinery to respond to local chemical cues that guide its axon along specific pathways. Once the axon arrives in the appropriate target area, however, it must still choose among a variety of potential targets. Much of this selection process probably involves chemical recognition between the appropriate pre- and postsynaptic cells. A further refinement of

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synaptic connectivity frequently occurs which is often dependent on the pattern of neural activity in the system.

The phenotype of a neurone is manifest not only by the transmitter substances it synthesizes and releases but also by the detailed pattern of inputs it receives from other neurones and the outputs it provides to others. Certain aspects of this phenotype may be determined by the lineage history of the neuroblast that gave rise to the neurone, while other characteristic features are known to be influenced by the neurone's local environment. Despite the fact that many of the underlying developmental mechanisms are similar in different neuronal systems, each system is likely to emphasize a distinct subset of these mechanisms. By examining an individual system in detail, one can determine which mechanisms are critical and how they contribute to the ultimate specificity and precision of connections in that system. A comparison of several different systems may then lead to a better understanding of why different mechanisms are emphasized in different systems.

The monosynaptic stretch reflex

This laboratory has been studying the development of the stretch reflex in the spinal cords of frogs and chickens. This reflex pathway constitutes the most direct connection between sensory and motor neurones in the central nervous system; it involves only one set of synaptic connections. Its afferent side consists of muscle sensory neurones that innervate muscle spindles in the periphery and are highly sensitive to changes in muscle length. Their central axons enter the spinal cord *via* the dorsal roots and then send collateral fibres ventrally into the spinal grey matter where they make monosynaptic, excitatory connections with motoneurones. The motoneurones, in turn, project directly back to the same muscle supplied by the spindle afferent fibres or to other, synergistic muscles. The effect of this reflex arc is to help maintain a muscle at an appropriate length. If the load on the muscle increases, its length increases as well, producing a volley of impulses in the spindle afferent fibres. The central axons of these fibres provide increased excitation to the motoneurones that supply the muscle, increasing its tension and thereby resisting the movement caused by the increased external load.

If this reflex is to operate effectively, spindle afferent fibres must excite only the correct subset of motoneurones. The high degree of specificity of these connections was first studied at the level of single motoneurones in cats by Eccles and his collaborators (Eccles *et al.* 1957, reviewed in Burke and Rudomín, 1977), and is now known to exist in most other vertebrates as well, including frogs (Tamarova, 1977; Frank and Westerfield, 1982*a*) and birds (Eide *et al.* 1982; Lee *et al.* 1988). Muscle spindle afferent fibres generally make their strongest projections to motoneurones supplying their own muscle (homonymous connections) or synergistic muscles (acting on either the same or a different joint). Much weaker connections are made with motoneurones supplying functionally unrelated muscles.

Although the central axons of muscle spindle afferent fibres arborize only in a

specific region of the spinal cord (Brown, 1981; Lichtman *et al.* 1984), they are nevertheless in an anatomical position to make contact with a large number of different types of motoneurones. Groups of functionally unrelated motoneurones are often adjacent to each other in the spinal cord and can have overlapping dendritic arborizations (Lichtman *et al.* 1984). For the stretch reflex to function appropriately, spindle afferent fibres must be capable of distinguishing among different types of potential targets within a common target area in the spinal cord. How is this recognition achieved during development?

Normal development of the stretch reflex

One possible strategy is that connections between different functional groups of muscle sensory and motor neurones are made at different times during development. Those neurones supplying one set of muscles could establish connections with each other first, and then become refractory to further synaptogenesis. Neurones supplying another muscle group could then become interconnected only after the first group became refractory. If time of development was important in the specification of sensory neurones, one might expect different types of neurones to be born (become postmitotic) at different developmental times. However, we find that sensory neurones supplying the triceps muscles in bullfrogs are generated simultaneously with those neurones supplying other forelimb muscles and cutaneous targets. Apparently the time a sensory neurone becomes postmitotic does not specify either its peripheral target or its sensory modality. Physiologically, spindle afferent fibres supplying the triceps brachii muscles in the bullfrog establish monosynaptic connections with triceps, but not pectoral or subscapular, motoneurones during the same developmental period as afferent fibres supplying these other, non-triceps muscles make connections with their own motoneurones (Frank and Westerfield, 1983). In the developing chick embryo, synaptic connections mediating the stretch reflex in different muscle groups are also made during the same time period (Lee *et al.* 1988).

Moreover, in both chickens and frogs, the specificity of these connections is apparent from the outset. From the earliest time that monosynaptic inputs from muscle afferent fibres can be recorded in motoneurones, these afferent fibres are found to connect only with appropriate targets (Frank and Westerfield, 1983; Lee *et al.* 1988). Anatomical studies show that physical contact between these neurones is established at the same time that monosynaptic connections can be detected electrophysiologically, so there is no reason to postulate a 'silent' period during which initially imprecise connections are re-arranged (Jackson and Frank, 1987; Davis *et al.* 1989; H. R. Koerber and B. Mendelson, unpublished observations). This initial specificity is similar to that seen in the development of motor innervation of muscle, where motoneurones specified to supply particular muscles project almost unerringly to their appropriate targets (Landmesser, 1980). But it stands in sharp contrast to the development of synaptic connections in the visual system, where synaptic activity plays an important part in establishing the normal,

adult pattern of connections. This observation provided the first clue that the mechanisms underlying the development of synaptic connections mediating the stretch reflex might be different from those used in the visual system.

Peripheral specification of sensory neurones

By analogy with the development of motoneuronal projections to muscles, sensory neurones might already be committed to supply a particular peripheral target at the time they first grow out from the dorsal root ganglia (DRGs). An alternative idea is that an initially uncommitted sensory neurone could become specified by its extrinsic environment. Le Douarin and her colleagues (Le Douarin, 1982) have shown that the phenotype of neural crest cells (which, of course, include sensory neurones) is strongly influenced by their final location in the body rather than their original position. Neural crest cells transplanted before they move away from the neural tube to novel locations along the neuraxis migrate to locations in the body that are appropriate for their new, transplanted location and they develop a phenotype that is also appropriate for this location. Although these experiments examined the fate of autonomic rather than sensory neurones, it seemed possible that a similar result might apply to sensory neurones as well.

We tested this idea by replacing the single brachial DRG (which normally provides the entire sensory innervation of the forelimb) in developing tadpoles with one or two DRGs transplanted from mid-thoracic levels (Smith and Frank, 1987). Whenever the transplantation was made sufficiently early for the transplanted ganglion to innervate muscle spindles in the forelimb, these foreign sensory afferent fibres also established monosynaptic connections with the appropriate brachial motoneurones in the spinal cord. That is, foreign sensory neurones supplying muscle spindles in the triceps brachii muscle projected strongly to triceps motoneurones but only weakly or not at all to subscapular or pectoral motoneurones, just as in normal frogs. The same result was obtained when the thoracic DRG was left in its original location but its peripheral axons were made to innervate the forelimb rather than their normal targets in the thorax (Frank and Westerfield, 1982*b*; Smith and Frank, 1988*a*). In that case, axons from these thoracic sensory neurones projected into the grey matter of the brachial spinal cord (something they never do in normal frogs) and established specific connections with the now functionally appropriate brachial motoneurones. Thus, the peripheral targets of these neurones seem to play a major role in determining both their target area within the spinal cord and the particular subpopulation of spinal motoneurones to which they project.

An interesting aspect of these experiments comes from the fact that not only the origin but also the *number* of sensory afferent fibres was changed. Normally the triceps muscle is provided with 30–40 spindle afferent fibres to its medial head and 15–20 fibres to the combined internal and external heads. But after transplantation or rerouting of thoracic DRGs, triceps muscles frequently received only 1–15 spindle sensory fibres. Sensory innervation to other forelimb muscles was also

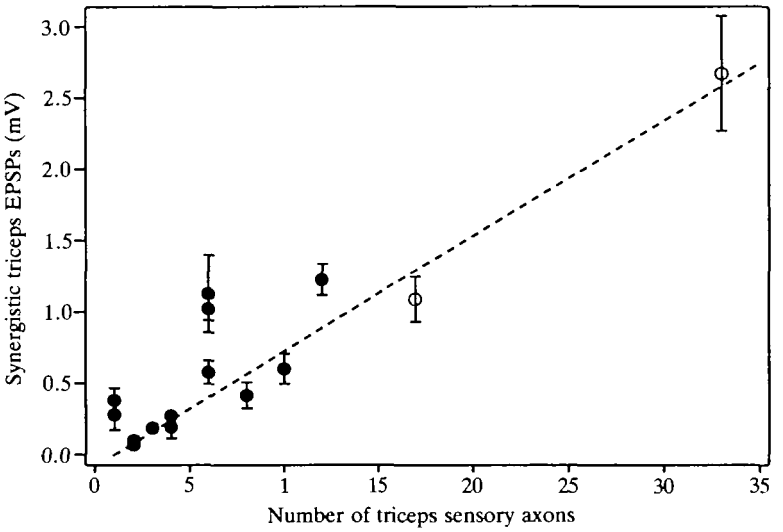

Fig. 1. Reduced numbers of triceps muscle spindle afferent fibres do not establish abnormally large synaptic projections to triceps motoneurones. The amplitude of the composite EPSP evoked in synergistic triceps motoneurones by stimulation of sensory afferent fibres in the medial or internal+external triceps muscle nerve is plotted against the number of afferent fibres in that nerve. The open symbols are for medial and internal+external triceps nerves in normal frogs, while filled symbols represent the results from frogs in which a reduced number of afferent fibres supplied the triceps muscles. This reduction was produced by removal of the brachial dorsal root ganglion (DRG) in young tadpoles, allowing an adjacent, thoracic DRG to supply the forelimb. The number of muscle afferents was measured electrophysiologically by recording from the dorsal root (see Mendelson and Frank, 1989, for details). The line has been drawn through the normal data to facilitate comparison; experimental data points falling on the line would represent no increase in the strength of the connections made by individual afferent fibres onto triceps motoneurones. Error bars in this and other figures represent 1 S.E. of the mean.

correspondingly reduced. Yet the reflex connections made by these foreign afferent fibres were as precise as in normal frogs (Frank and Mendelson, 1990). Furthermore, the synaptic strength of these connections was also approximately normal (Mendelson and Frank, 1989). In normal frogs, medial triceps sensory afferent fibres evoke, on average, a composite 2.7 mV excitatory synaptic potential (EPSP) in the synergistic internal/external triceps motoneurones and the reciprocal synergistic connection between internal/external triceps sensory fibres and medial triceps motoneurones is 1.1 mV. This means that each triceps sensory fibre elicits an EPSP in each synergistic triceps motoneurone of, on average, 65–80 μV. As shown in Fig. 1, the synergistic EPSPs elicited by the reduced number of triceps sensory fibres in the experimental frogs were of similar amplitude. That is, these fibres did not react to the paucity of sensory innervation of the brachial spinal cord by making an abnormally large number of synaptic connections. Apparently the peripheral influence that determines the *specificity* of

the central connections of muscle sensory neurones can determine the *magnitude* of these projections as well.

Influence of patterned neural activity

As mentioned earlier, in some parts of the central nervous system patterned neural activity plays an important part in refining the initial set of synaptic connections. In the visual system, for example, ocular dominance, orientation specificity and the precision of the retino-tectal map all depend on visual experience and can be disrupted by blocking normal patterns of activity. Muscle spindle afferent fibres are sensitive to stretch and neurogenic muscle contraction occurs during the time that sensory-motor connections are forming in the spinal cord. Therefore, the correlated and/or anti-correlated patterns of activity in muscle sensory and motor cells might be used by the developing nervous system to ensure that correct groups of neurones establish functional synaptic contacts with each other. We have used two experimental approaches to examine this possibility.

In one set of experiments, the normal pattern of motor innervation of the forelimb was disrupted in tadpoles by resection of the brachial ventral root (Frank, 1990). The operation was performed at stages XIV–XVII (Taylor and Kollros, 1946), well after both sensory and motor axons have innervated peripheral targets but just before the muscle sensory neurones begin to form their central connections with motoneurones. Active movement of the limb was therefore blocked during much of the time that these connections were forming. In fact, even after motoneurones reinnervated the limb, the pattern of reinnervation was non-specific. Fig. 2 illustrates the results of retrograde labelling of the triceps motoneurones with horseradish peroxidase (HRP) in one of these frogs. Labelled triceps neurones are tightly clustered on the normal side of the animal, but are scattered throughout the entire extent of the brachial motor column after ventral root resection. As a result, the post-metamorphic frogs were unable to move their affected forelimbs. Thus, synaptogenesis occurred in the absence of any organized limb movement.

The central connections of triceps muscle afferent fibres in these animals were determined by making intracellular recordings from brachial motoneurones in the region of the spinal cord where triceps motoneurones are normally located. As expected from the non-specific motor reinnervation, relatively few of these neurones now projected to the triceps muscle. Furthermore, the pattern of triceps sensory innervation was functionally non-specific. Many motoneurones that now projected to the triceps muscle received no triceps sensory input, while some subscapular and pectoral motoneurones (which normally receive little or no triceps input) *were* supplied by triceps inputs.

The connections of triceps sensory fibres were not random, however, because those afferent axons that supplied the medial and combined internal/external heads of the triceps muscle innervated the *same* subpopulation of brachial

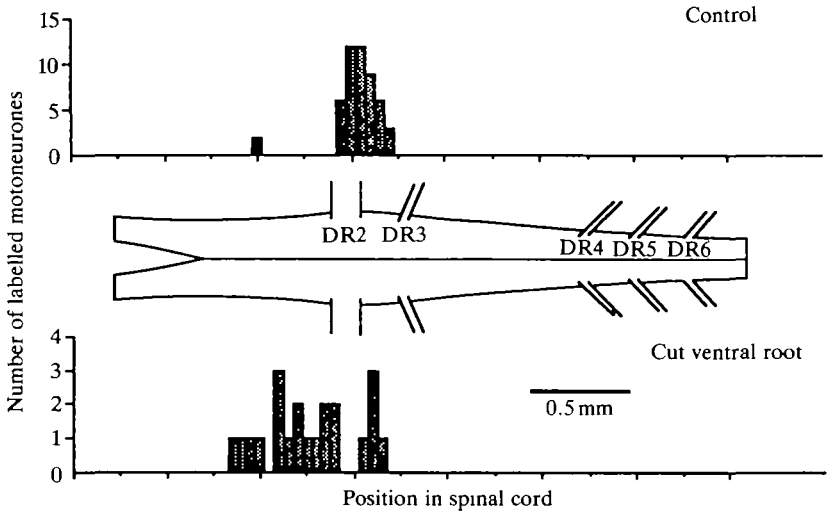

Fig. 2. Non-specific regeneration of triceps motoneurones in a juvenile bullfrog after resection of the ventral root on one side at stage XVI. Triceps motoneurones were labelled retrogradely on both sides with horseradish peroxidase (HRP) and their positions recorded from serial transverse sections. A drawing of the dorsal view of the spinal cord is included for orientation and shows the positions of the dorsal roots (DR). The ordinate represents the number of labelled motoneurones in each section.

motoneurones. In normal frogs, triceps motoneurones receive strong inputs from both classes of triceps afferents (medial *and* internal/external) whereas most other motoneurones in this region of the spinal cord receive very little input from either triceps class. This is illustrated in Fig. 3A, which shows the strong correlation between the two types of triceps sensory inputs in individual motoneurones. As illustrated in Fig. 3B, a strong correlation was also observed for frogs with non-specific motor reinnervation of their forelimbs. Any motoneurone that received strong input from one class of triceps sensory neurones received strong input from the other class as well, no matter whether that motoneurone happened to reinnervate the triceps muscle or not.

The correlation of sensory inputs to individual motoneurones was specific for triceps inputs. As shown in Fig. 4, there was no correlation for either normal or experimental frogs between medial triceps input and input from the combined subscapular and pectoral muscle afferent fibres. Therefore, the observed correlation between the two triceps inputs cannot be explained by the quality of the intracellular recordings or because some motoneurones were receptive to all sensory inputs while others were not. The implication is that both classes of triceps sensory fibres selectively innervated a distinct subpopulation of brachial motoneurones, presumably those motoneurones that had originally supplied the triceps muscle. Muscle afferents can make this discrimination in the absence of stretch-

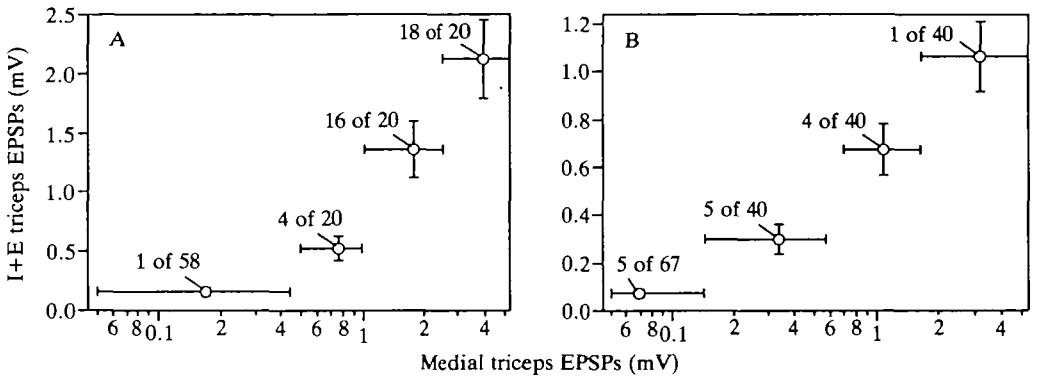

Fig. 3. Correlated inputs of medial and internal+external (I+E) triceps sensory afferent fibres to brachial motoneurones in normal frogs (A) and in frogs with non-specific motor reinnervation of the forelimb (B). In this and the subsequent figure, each point represents the average triceps input to a number of individual motoneurones; horizontal bars indicate the range of medial triceps sensory input to each group. The numbers next to each point indicate what fraction of the total number of motoneurones in each group supplied the triceps muscle. In normal frogs, all but one of the triceps motoneurones received significant triceps sensory input, but in the experimental frogs, many 'triceps' motoneurones received very little triceps sensory input. The connections were thus functionally inappropriate. In both groups of frogs, however, motoneurones with large inputs from one set of triceps afferent fibres had large inputs from the other set as well, while those with small inputs from one group also had small inputs from the other. The two groups of afferent fibres thus selected the same subpopulation of motoneurones, even in frogs with non-specific motor reinnervation. Horizontal axes have been plotted logarithmically simply to separate the small-amplitude points from each other.

evoked activity in spindle afferent fibres, arguing for a precise molecular recognition between appropriate synaptic partners.

In a second approach to determining the role of patterned activity in the establishment of the stretch reflex, we blocked stretch-evoked responses in spindle afferent fibres by chronically paralyzing developing chick embryos with curare applied daily to the chorioallantoic membrane, as described by Pittman and Oppenheim (1979). Chick embryos are more suitable for these experiments than tadpoles because they can be completely paralyzed for 1–2 weeks (between stages 28 and 39, Hamburger and Hamilton, 1951), which is the period of synaptogenesis between spindle afferent fibres and motoneurones supplying hindlimb muscles (Lee *et al.* 1988). Curare applied in this fashion also blocks most of the spontaneous bursts of activity in motoneurones at these stages (Landmesser and Szente, 1986) and should therefore disrupt correlated patterns of activity in muscle sensory and motor cells.

Intracellular recordings made from wing and hindlimb motoneurones in curare-treated embryos showed that the pattern of synaptic connections was essentially normal. Representative traces from a normal and a curare-treated embryo are

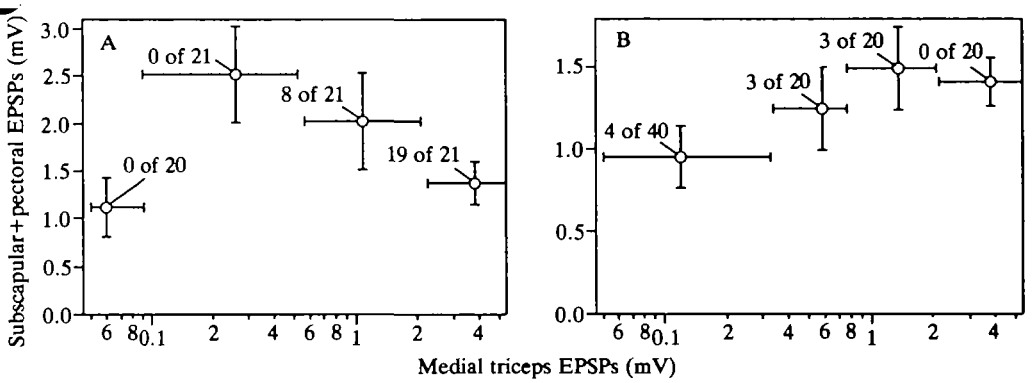

Fig. 4. Inputs from medial and subscapular+pectoral sensory afferent fibres to brachial motoneurones are *not* correlated with each other, either in normal frogs (A) or in frogs with non-specific motor reinnervation (B). Motoneurones with little input from medial triceps fibres were just as likely to receive significant input from subscapular+pectoral afferent fibres as motoneurones with large medial triceps sensory input. This control experiment shows that the results presented in Fig. 3 are not an artefact but instead that triceps sensory afferents selectively innervated a distinct subpopulation of brachial motoneurones.

shown in Fig. 5. Just as in normal embryos, homonymous inputs in embryos blocked for 10 days with curare were strong while inputs to functionally unrelated motoneurones were weak. In certain cases, disynaptic inhibition to antagonistic motoneurones was observed (lower left trace in each group of records), just as in normal animals.

These results are summarized in Fig. 6. The figure shows the correlation between corresponding EPSPs in normal and paralyzed embryos. Each point represents the average monosynaptic input evoked in a particular class of motoneurone by stimulation of a particular class of muscle sensory neurone. Homonymous connections are shown with filled symbols. Whenever a particular class of EPSPs was large in normal chicks, it was also large in the paralyzed embryos, and the same was true for weak connections. The only difference in the connectivity we have noticed to date (and for which we have as yet no explanation) is that EPSPs in the paralyzed embryos tend to be larger than normal, as is obvious from the slope of the line in Fig. 6. These results are therefore in full agreement with those from the tadpoles with resected ventral roots, and they argue strongly for a recognition between appropriate classes of muscle sensory and motor neurones that is not dependent on patterned neuronal activity.

Possible mechanisms

A straightforward interpretation of all these results is that peripheral targets impose an *instructive* molecular influence on the sensory neurones that supply them. According to this interpretation, a sensory neurone would be uncommitted

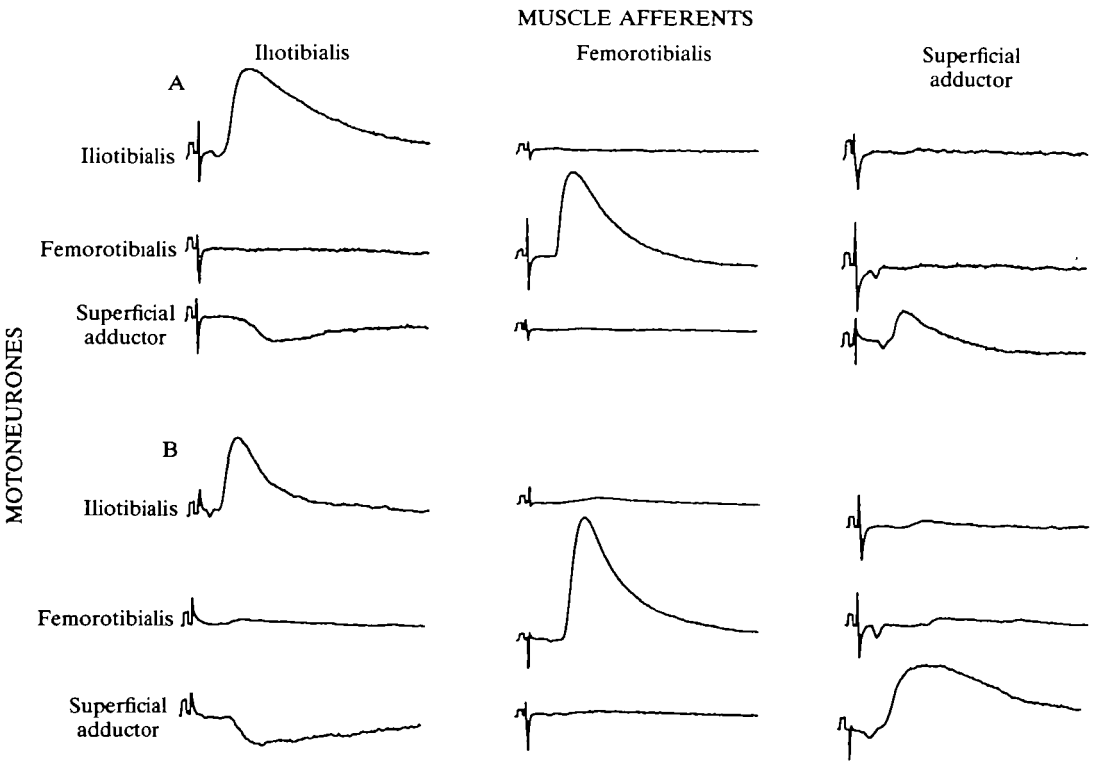

Fig. 5. Intracellular recordings from lumbosacral motoneurones in two stage 39 chick embryos, normal (A) and paralyzed for 10 days with curare (B). For each embryo, EPSPs evoked by stimulation of three different groups of muscle afferent fibres were recorded in three different motoneurones. In both normal and experimental embryos, homonymous inputs elicited large, monosynaptic EPSPs; functionally unrelated inputs were small. One group of antagonistic muscle afferents provided disynaptic inhibition (lower left trace in each group). Muscle afferent fibres therefore make highly selective connections with motoneurones even when limb movements are blocked with curare. Calibration pulses in each trace are 0.5 mV and 2 ms.

as to a particular phenotype at the time its axon first grows out from a DRG. Because many sensory neurones project to their peripheral targets before they establish synaptic connections within the spinal cord (Ramon y Cajal, 1929; Windle, 1934; Windle and Baxter, 1936; Vaughn and Grieshaber, 1973; Smith, 1983; Smith and Frank, 1988*b*), there would be sufficient time for the target to produce some molecular change in the sensory ending that could be communicated back to the nucleus by retrograde axoplasmic transport. Depending on which target the sensory neurone happened to innervate, it could then be specified to make the appropriate central connections.

The fact that many sensory neurones die during normal development provides another way of interpreting these data. Each DRG might produce all possible types of sensory neurone, each one completely determined to project to a

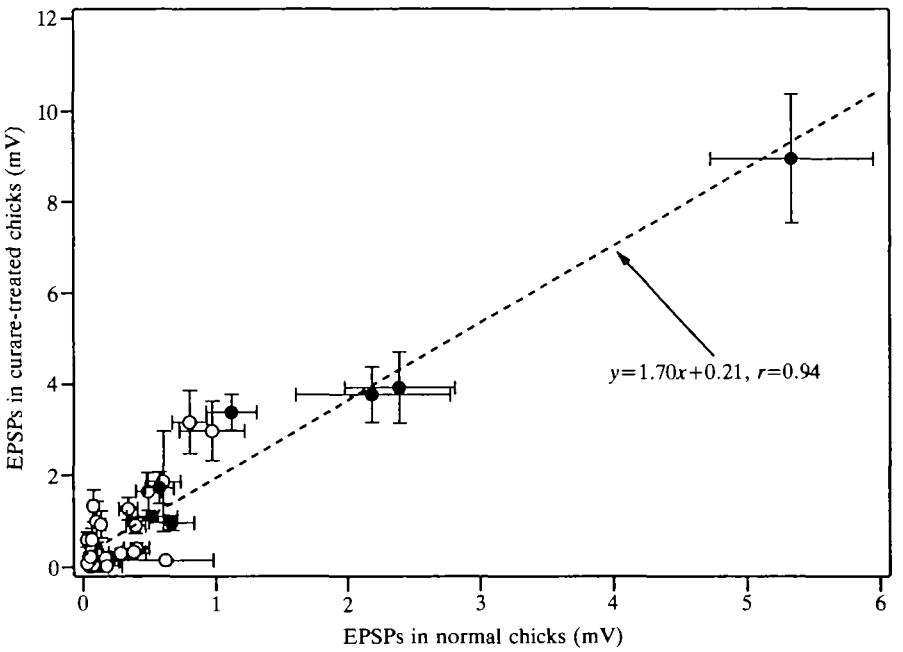

Fig. 6. Comparison of average synaptic inputs to motoneurones in normal and curare-treated chick embryos. The monosynaptic excitatory inputs to six kinds of motoneurones from six kinds of muscle afferent fibres are shown as 36 points; the ordinate and abscissa represent EPSP amplitudes in paralyzed and normal embryos, respectively. Homonymous connections are shown as filled symbols, other connections are open symbols. The line is a least-squares fit of the data and shows that, on average, each connection was 1.7 times larger in paralyzed embryos. Nevertheless, the pattern of connectivity is virtually normal, as indicated by the high correlation coefficient, *r*.

particular peripheral target and to a particular target population of spinal neurones. If a specific peripheral target (such as the medial triceps brachii muscle) did not exist at one or more segmental levels (as in the thorax), the sensory neurones determined to innervate that target would simply die. A similar interpretation of selective cell death can be applied to the original neural crest transplantation experiments. The novel phenotype of crest cells transplanted to a novel axial location might simply result from the selection of a distinct subpopulation. The effect of the periphery could thus be *permissive* rather than *instructive*.

It is hard to imagine how selective cell death could provide a complete explanation of our results, however. In the extreme form, one would need to postulate that every DRG in the frog, for example, produces a sufficient number of neurones to supply every peripheral target at every segmental level. Although many sensory neurones do die during development, the fraction is probably no larger than 50–67 % (Prestige, 1965). Simple arithmetic shows that this number of neurones would be insufficient.

A third alternative is that the real answer lies somewhere between the two

extremes of complete instructive and complete permissive determination. There is some evidence that different groups of sensory neurones are already different from one another at the time they begin to grow processes into the periphery. Sensory neurones in the mesencephalic nucleus of the developing chicken brain (TMN neurones) survive and grow neurites when cultured in the presence of crude muscle extracts, but do not survive in minimal media supplemented only with nerve growth factor (NGF) (Davies, 1986). These TMN neurones normally project exclusively to muscle spindles rather than skin (Manni *et al.* 1965). In contrast, sensory neurones in the ventrolateral part of the trigeminal ganglion grow well in the presence of NGF but do not survive in muscle-conditioned medium. These trigeminal neurones supply cutaneous but not muscle targets in the periphery (Noden, 1980*a*,*b*). DRGs might contain both populations of neurones; if muscle spindles were present in the periphery, the subpopulation analogous to TMN neurones would survive but otherwise it would disappear. This could explain why we have been unable to get thoracic sensory neurones (which normally do not innervate muscle spindles in frogs) to provide a functional innervation of muscle spindles in the forelimb if their peripheral axons are rerouted *after* the period of normally occurring cell death (S. C. Mears and E. Frank, unpublished observations).

In summary, these studies of the developing stretch reflex have shown that a highly ordered set of synaptic connections can develop without recourse to patterned neuronal activity. Nevertheless, the central connections made by muscle spindle afferent neurones are strongly influenced by the peripheral targets these neurones supply. This influence is likely to be mediated *via* some molecular influence of the target on the sensory neurones. An exciting goal for further research will be to determine precisely which aspects of sensory neuronal phenotype are determined shortly after a neurone is born and simply selected by a peripheral target and which are actively specified by the target.

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