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DEVELOPMENT OF SENSORY–MOTOR SYNAPSES IN THE SPINAL CORD OF THE FROG

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SUMMARY

1. The development and specificity of monosynaptic sensory–motor synapses were studied in the brachial spinal cord of bullfrog tadpoles. Intracellular and extracellular recordings were made from motoneurones innervating several different muscles of the forelimb. Excitatory synaptic potentials (e.p.s.p.s) were elicited by stimulation of various peripheral muscle nerves.

2. Sensory and motor axons in the triceps brachii muscle nerves were electrically excitable at stage XIII, the earliest stage studied. Their conduction velocities were 0·2–0·4 m/s. These velocities increased during subsequent development so that by stage XXII they were approximately 5 m/s.

3. Before stage XVII, synaptic potentials evoked in motoneurones by stimulation of the triceps sensory fibres had a long central latency and fatigued easily. These potentials were probably mediated polysynaptically.

4. At stage XVII, the first short-latency triceps synaptic potentials appeared. They had central latencies of less than 3 ms and represented the direct, monosynaptic input from muscle sensory cells on to motoneurones. During subsequent development the percentage of triceps motoneurones innervated by triceps sensory fibres increased, while the number of long-latency polysynaptic inputs decreased.

5. Both the electrical and chemical components, characteristic of these monosynaptic e.p.s.p.s in adult frogs, were prominent from the time the e.p.s.p.s first appeared.

6. The pattern of innervation of brachial motoneurones by triceps sensory afferents was specific from the beginning. Triceps sensory fibres innervated most triceps motoneurones but very few subscapular or pectoralis motoneurones, just as in adult frogs. At no time were there appreciable numbers of 'aberrant' connexions.

7. The developmental time course of several different classes of sensory–motor connexions was similar. Thus the synaptic specificity of this system cannot be explained by a differential timing of synaptogenesis.

INTRODUCTION

The elucidation of mechanisms responsible for the formation of specific connexions among nerve cells is one of the major areas of investigation in neurobiology. The development of synaptic connexions has been studied in many areas of the nervous

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system including the spinal cord. In adults, synapses between muscle sensory neurones and spinal cord motoneurones are highly ordered (Eccles, 1957), and their precise organization has been demonstrated in both mammals (Eccles, Eccles & Lundberg, 1957) and amphibians (Frank & Westerfield, 1982*a*). In general, muscle afferent axons project strongly to motoneurones innervating their own muscles (homonymous connexions), but weakly or not at all to motoneurones innervating functionally unrelated muscles.

The development of this reflex pathway has been studied in spinal cords of the cat (Skogland, 1960*a*,*b*; Wilson, 1962; Eccles & Willis, 1963; Eccles, Shealy & Willis, 1963; Naka, 1964*a*,*b*; Eccles & Willis, 1965; Kellerth, Mellstrom & Skogland, 1971; Mellstrom, 1971*a*,*b*), rat (Saito, 1979) and chick (Provine, Sharma, Sandel & Hamburger, 1970; Sharma, Provine, Hamburger & Sandel, 1970; Provine & Rogers, 1977). However, in none of these studies has the pattern of synaptic connexions, at the time they first form, been assayed by intracellular recording and stimulation of single muscle nerves. Only in this way can one determine directly the specificity of the first functional connexions between sensory and motor neurones.

We describe here the functional development of monosynaptic connexions between muscle sensory and motor neurones in the spinal cord of bullfrog tadpoles. Our results demonstrate that at the time of their initial formation, synapses between muscle sensory and motor neurones become functional with the adult pattern of connectivity.

METHODS

Animals. Larval and juvenile bullfrogs (*Rana catesbeiana*) of both sexes were studied. Frogs were kept at room temperature and fed trout chow (Purina) 3 times weekly. Tadpoles were kept in tanks with recirculating water at 25 °C. The water was a diluted Holtfreter solution ((mM): Na^+, 4·7; K^+, 0·05; Ca^{2+}, 0·07; Cl^-, 4·7; HCO_3^-, 0·2) and was changed daily. An agar-based tadpole food, kindly provided by Dudley Culley, Louisiana State University, was constantly available. Tadpoles were staged according to the criteria of Taylor & Kollros (1946).

At stages XIII–XIV, the earliest stage used, the digits of the hand have just become well separated, and simple reflex withdrawals of the limb can be elicited by cutaneous stimulation. At stages XVII–XVIII, the hand appears similar to an adult hand, and metamorphosis begins. The forelimbs erupt through the body wall at stage XX, and they begin to be used in crawling and swimming. After this stage, the tail is progressively reabsorbed until it disappears at stage XXV, the end of larval development.

Dissection. Spinal cords were isolated using procedures similar to those described earlier (Frank & Westerfield, 1982*a*; Westerfield & Frank, 1982). In most preparations the spinal cord was hemisected longitudinally near the dorsal–ventral mid line. Individual muscle nerves in the arm were dissected and prepared for stimulation. These included branches to the internal, external and medial heads of the triceps brachii muscles, the nerves supplying the pectoral and subscapular muscles, and the ulnar and radial nerves which innervate all the muscles in the forearm and hand. The preparation was then transferred to an experimental chamber for recording. Nerves were stimulated with suction electrodes. The temperature of the bath was maintained at about 14 °C and the preparation was perfused with fresh, oxygenated saline of the following composition (mM): Na^+, 116; K^+, 2; Ca^{2+}, 1·8; Cl^-, 122; glucose, 15; HEPES buffer, 5, pH 7·2.

Test solutions were also used which contained 0·3–0·5 mM-Ca^{2+} and 7–9 mM-Mg^{2+}. Solutions were completely changed within 30 min, as judged from changes in the intracellular potentials.

Electrophysiology. Motoneurones were impaled with glass micro-electrodes filled with 3 M-KCl or 0·5 M-K citrate with 0·5 % Fast Green added to make the tips visible. After stage XVI, motoneurones were identified by antidromic activation. Before this stage, antidromic action potentials were not propagated into motoneuronal somata, presumably because of the relatively small diameter of the immature, non-myelinated motor axons.

Intracellular recordings from motoneurones in tadpoles before stage XIX were difficult, and we frequently used electrodes with resistances of 100–200 MΩ. There was no obvious correlation between synaptic potential amplitudes and resting potentials in cells whose resting potential was −40 mV or greater; we therefore used −40 mV as the minimum acceptable resting potential. Measurements of amplitude and latency were made from two to twenty individual responses using a signal averager (Dagan) to reduce the effects of electrode noise and spontaneous synaptic activity. Extracellular field potentials were negligible before stage XX; after that stage, we subtracted the field potential from the intracellular record electronically after withdrawing the pipette from the cell. In some experiments, ventral or dorsal roots were acutely sectioned and placed in tight-fitting suction electrodes for extracellular recording.

Measurements of muscle sensory input to heteronymous and unrelated motoneurones are straightforward since the muscle nerve can be stimulated so that all myelinated sensory axons are activated. However, homonymous sensory inputs (for example, medial triceps sensory axons on to medial triceps motoneurones) are difficult to measure for two reasons. First, sensory and motor axons have similar electrical thresholds, so that all the sensory axons cannot be activated without contaminating the recording with the antidromic impulse. Secondly, homonymous motoneurones are electrically coupled with each other. Thus, even below the threshold for antidromic activation of the cell being studied, the synaptic input from sensory cells is contaminated by electrical coupling potentials from the antidromic impulses in other motoneurones. These problems have been discussed in detail elsewhere (Frank & Westerfield, 1982*a*; Westerfield & Frank, 1982). Because of these difficulties, we have not included measurements of homonymous sensory–motor connexions.

The results presented here were obtained from 78 tadpoles; intracellular records were obtained from 790 motoneurones. The results from adult animals are from the experiments described in Frank & Westerfield (1982*a*).

RESULTS

I. Appearance of monosynaptic sensory–motor connexions

Presence of sensory and motor axons in the muscle nerve. Before studying the development of sensory–motor synapses within the spinal cord, we needed to determine when these axons were present in the muscle nerve and the conduction times required for these impulses to propagate into the spinal cord. The measurements were obtained by making extracellular recordings from the distal portions of cut dorsal and ventral roots.

Sensory and motor axons were present in the triceps muscle nerve by stage XIII, the earliest stage studied. Peripheral conduction times ranged from 5 to 10 ms. This corresponds to a conduction velocity of 0·2–0·4 m/s, appropriate for non-myelinated axons. As development proceeded, conduction times decreased so that by stage XXII sensory impulses reached the central end of the dorsal root in 1–1·5 ms, corresponding to a conduction velocity of approximately 5 m/s. This progression is shown in Fig. 1. After this stage, the conduction time remained constant, although the conduction distance and hence velocity continued to increase. Before stage XXII, there was a range of conduction times (indicated by arrows in Fig. 1), consistent with the idea that some but not all axons were myelinated. A similar progressive shortening in conduction times was seen for triceps motor axons, recorded in the ventral root.

Intracellular recordings. The development of synaptic connexions between sensory and motor neurones innervating the triceps muscles was studied by stimulating the muscle nerve while recording intracellularly from the motoneurones. Examples of intracellular recordings at various developmental stages are shown in the right-hand side of Fig. 2. At stage XIV, the earliest stage studied with intracellular electrodes,

most cells received no input from triceps sensory axons (Fig. 2). Although we could not identify motoneurones antidromically at this stage (see Methods), one-third of the motoneurones in the triceps region of the spinal cord innervate triceps muscles (Frank & Westerfield, 1982*a*), so approximately six or seven of the twenty cells were probably triceps motoneurones. In the few cells that did receive triceps input, the synaptic latency varied from 25 to 45 ms, and fluctuated by several milliseconds from trial to trial. Moreover, the potentials disappeared in response to repetitive stimulation,

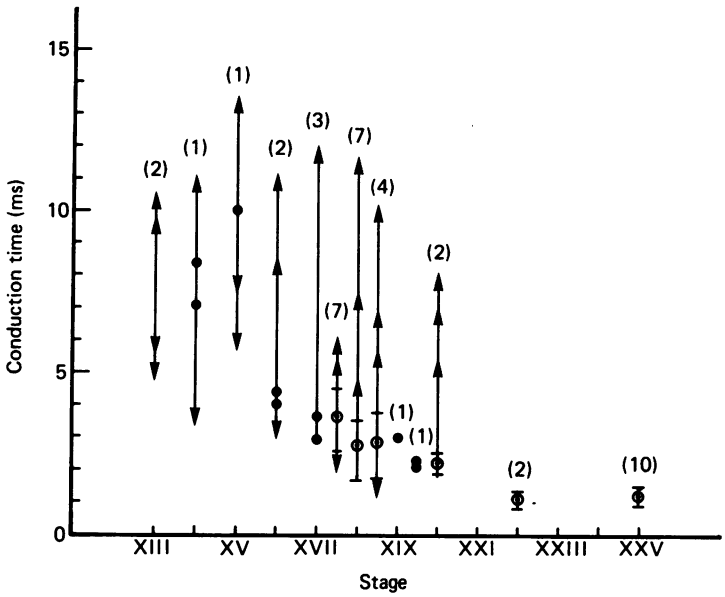

Fig. 1. Peripheral conduction times of triceps muscle sensory axons in developing tadpoles. Sensory impulses were recorded with a suction electrode from the cut peripheral end of the second dorsal root. The triceps nerve was stimulated peripherally (0·15–0·5 ms square pulses) with a second suction electrode. Conduction times were measured from the beginning of the stimulus to the positive peak in the dorsal root record. At earlier stages (up to stage XX), several peaks were observed, indicating a range of conduction velocities. Major peaks in such cases are indicated by filled circles while the total range for each experiment is indicated by arrowheads. After stage XVIII, individual major peaks are indicated by their mean (open circles) ± 1 S.D. The number of animals tested at each stage is indicated in parentheses. There is a progressive shortening of conduction times during development, with a plateau of 1–1·5 ms by stage XXII.

even at 0·2 Hz. Since sensory impulses arrive at the spinal cord within 10 ms (arrow in Fig. 2, see below), it is probable that the synaptic potentials arose via a polysynaptic pathway.

By stage XVII, the proportion of triceps motoneurones with some form of synaptic input from triceps sensory axons was 33% (Fig. 2). In half of these cases, or 16% of the total population, the synaptic potentials began within 3 ms after the sensory impulses had reached the spinal cord. An example is shown to the right of the histogram. Because of this short central delay, these excitatory synaptic potentials (e.p.s.p.s) were probably mediated monosynaptically (see below).

During subsequent development, there was a progressive increase in the proportion

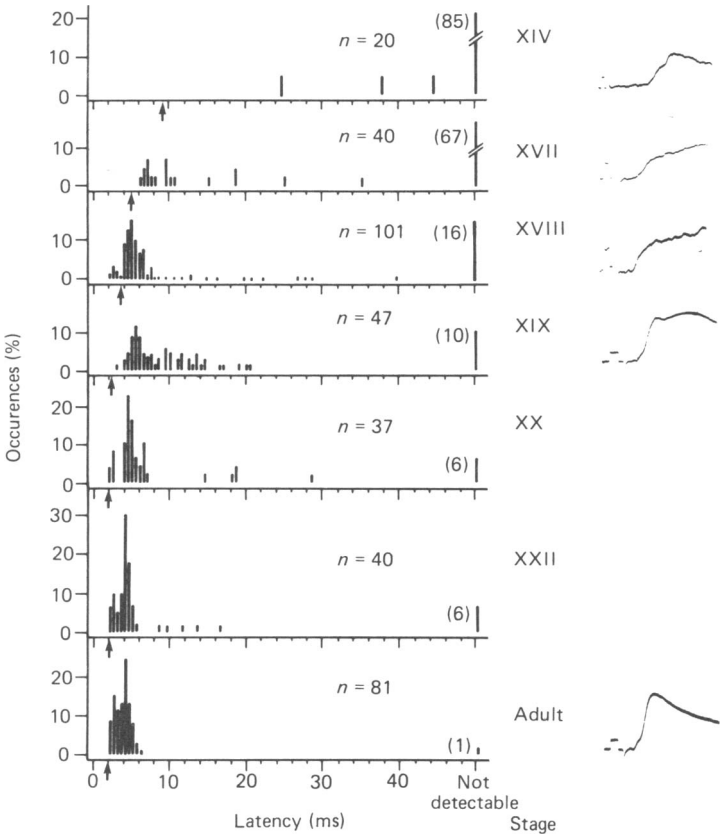

Fig. 2. Latency histograms of triceps sensory input to triceps motoneurones at various developmental stages. Motoneurones were identified by antidromic activation except at stage XIV (see Methods). Only heteronymous triceps e.p.s.p.s are included (medial triceps sensory axons on to internal–external triceps motoneurones and vice versa) because homonymous inputs are contaminated by electrical coupling potentials from antidromic activation of homonymous motoneurones (Westerfield & Frank, 1982). The time of arrival of sensory impulses is indicated by an arrow in each histogram; this time was determined from ventral root records like those in Fig. 4 (see text). The histograms include all components with amplitudes of 0·2 mV or greater; thus if a cell had both early and late inputs, both are indicated. The percentage of sensory–motor pairs that had undetectable responses is shown in the right-hand column, in parentheses. *n*, number of sensory–motor pairs tested at each stage. Examples of intracellular records of triceps e.p.s.p.s are shown to the right of several of the histograms. In these examples the ventral root was cut to ensure that only sensory inputs were activated. However, the responses seen in intact preparations were indistinguishable from those shown. Each trace is the average of three to ten individual responses. Calibration, 0·5 mV and 2 ms.

of triceps motoneurones with short latency (< 3 ms central delay) triceps input. At stage XIX, two stages after monosynaptic triceps e.p.s.p.s appeared, 50 % had short latency input; 86 % at stage XXII and 99 % in adults. These results show that the great majority of monosynaptic triceps sensory inputs to motoneurones are made at and after stage XVII.

Extracellular recordings. A potential disadvantage of intracellular recordings at

earlier developmental stages is that the number of cells sampled is relatively small and the identity of these cells cannot be determined by antidromic activation. To determine if there was a class of motoneurones that did receive monosynaptic input from triceps sensory axons at these early stages, we recorded from the cut central end of the second ventral root while stimulating the triceps muscle nerve. This ventral root contains the axons of all the brachial motoneurones, so the recordings give a measure of the average input these neurones receive from triceps sensory axons. The

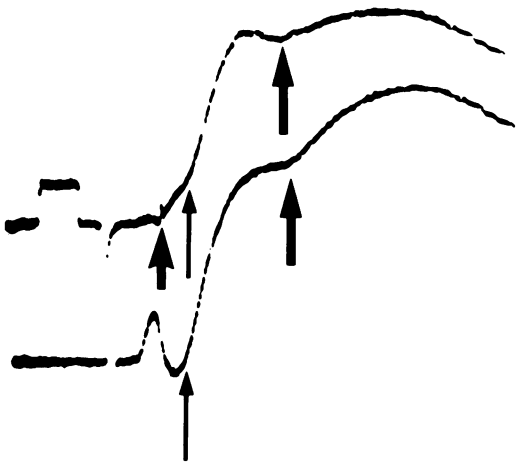

Fig. 3. Simultaneous intracellular and extracellular recordings from a motoneurone in a tadpole at stage XIX in response to stimulation of all branches of the triceps muscle nerve. The intracellular recording (upper trace, resting potential −58 mV) was made from a neurone within the triceps motor pool. The extracellular recording (lower trace) was made with a suction electrode from the cut central end of the second ventral root. The first positive peak in the extracellular record presumably represents the invasion of sensory axons in the vicinity of motoneuronal dendrites; its latency (2·4 ms) is within the range of peripheral conduction times measured in the dorsal root at this stage (refer to Fig. 1). The earliest component of the triceps e.p.s.p. recorded intracellularly (short, thick arrow; latency of 2·8 ms) occurs within 0·5 ms of the sensory impulses, and is presumably mediated electrically. The monosynaptic chemical component, seen in both recordings (long, thin arrows; latencies of 3·8 and 3·9 ms) has a central delay of 1·5 ms, at 14 °C. Finally, later, polysynaptic components are seen in both records (long, thick arrows; latencies of 10 ms). Each trace is the average of three to ten individual responses. Calibration, 0·5 mV for the intracellular record and 2 ms for both records.

various components in these recordings were identified by recording intracellularly from a brachial motoneurone while simultaneously recording extracellularly from the ventral root. Fig. 3 shows one pair of simultaneous intra- and extracellular recordings from a tadpole at stage XIX, a stage when monosynaptic sensory–motor connexions have already developed.

The earliest component in the ventral root record was a predominantly monophasic potential similar to that seen in records of sensory impulses in dorsal roots. It began only 1·5 ms after stimulation of the triceps nerve, and its positive peak had a latency of 2·4 ms. These values are within the range of peripheral conduction times for triceps sensory impulses recorded in the dorsal root (see Fig. 1). This first component thus represents the arrival of sensory impulses in the spinal cord. We have used its latency

as the best available indication of when sensory impulses reach the vicinity of motoneuronal dendrites.

The second component in the ventral root record (long, thin arrow in lower trace of Fig. 3) arose with a latency of 3·8 ms, very similar to the latency (3·9 ms) of the chemical component of the monosynaptic triceps e.p.s.p. recorded intracellularly (long, thin arrow in upper trace of Fig. 3). This corresponds to a central delay of 1·5 ms, in agreement with the value found by several investigators for the chemical component of monosynaptic sensory–motor e.p.s.p.s in adult frogs (Shapovalov, Shiriaev & Velumian, 1978; Alvarez-Leefmans, de Santis & Miledi, 1979; Shapovalov & Shiriaev, 1978, 1980; Westerfield & Frank, 1982). The earliest, electrically mediated, component of the intracellular e.p.s.p. (short, thick arrow in upper trace of Fig. 3; also see below) was not prominent in ventral root recordings made at any developmental stage, including adult animals.

Finally, in some of the ventral root records there are later components, such as the one indicated by the long, thick arrow in Fig. 3, corresponding to later components in the intracellular record. In contrast to the early components, these later ones were labile and could not be elicited at stimulus frequencies higher than about 0·2 Hz, suggesting they may be mediated polysynaptically. Moreover, any components with central latencies longer than approximately 3 ms are probably polysynaptic in this preparation (Tamarova, 1977; Shapovalov *et al.* 1978; Alvarez-Leefmans *et al.* 1979; Shapovalov & Shiriaev, 1980; Frank & Westerfield, 1982*a*). We have used this value of 3 ms central delay as the upper limit for monosynaptic e.p.s.p.s.

Ventral root recordings of triceps sensory input to brachial motoneurones made at several developmental stages are shown in Fig. 4. At stages XIII–XIV the major component in the ventral root began after a latency of 25–40 ms, corresponding to a central delay of 15–30 ms. No components were seen with latencies consistent with monosynaptic connexions. However, by stage XVII (and in one animal at stage XVI), earlier components were visible in some preparations with central delays less than 3 ms, suggesting that monosynaptic sensory–motor connexions were forming. Later, polysynaptic components were still prominent in many of the ventral root records, as in the intracellular traces (refer to Figs. 2 and 3). With further development, the early components became more prominent and the late components less so. These results are analogous to those obtained with intracellular recordings, and they suggest that few, if any, monosynaptic connexions between triceps sensory and motor cells appear before stage XVII.

II. Specificity of developing sensory–motor connexions

At the neuromuscular junction and in certain peripheral ganglia, synaptic connexions undergo a considerable remodelling during development (see Purves & Lichtman, 1980). Many early contacts are lost, and these rearrangements may aid in establishing the specificity of the adult pattern of connexions. In the adult bullfrog, triceps sensory fibres make strong projections to triceps motoneurones, but provide only weak input to several species of non-triceps motoneurones (Frank & Westerfield, 1982*a*). To determine if there were rearrangements of these connexions during

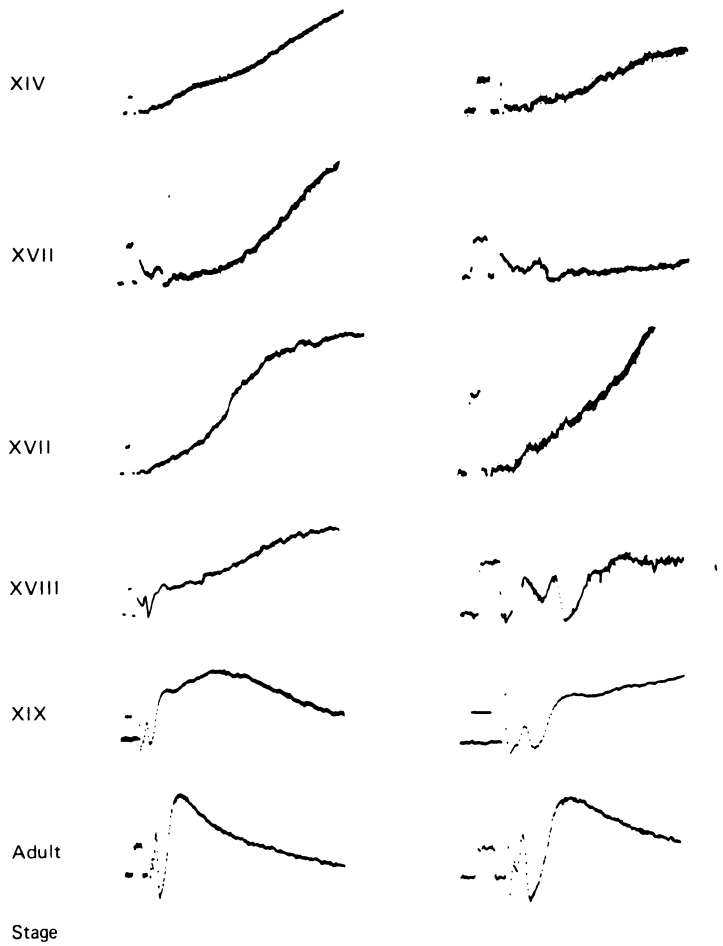

Fig. 4. Ventral root recordings of triceps sensory input to brachial motoneurones at various stages of development. Each response is shown at slow (left) and fast (right) sweep speeds. Before stage XVII, these records showed dispersed arrival of triceps sensory impulses into the spinal cord (compare with Fig. 1) and some late components which represent polysynaptic responses with latencies of 25–40 ms. In the upper stage XVII record, the dispersal of sensory impulses is sufficiently small that a distinct peak can be seen at 5·6 ms. This is followed by a slowly rising polysynaptic response beginning at about 23 ms, but there is no evidence for any monosynaptic triceps input to motoneurones in this experiment. In the lower stage XVII record, the sensory peak is followed within a few milliseconds by later components; the earliest of these represent monosynaptic inputs. At later stages, the monosynaptic component becomes more prominent while the later, polysynaptic components become less prominent. Each trace is the average of three to ten individual responses. The calibration pulse before the stimulus in each trace is 2 ms.

development, we compared the amplitudes of monosynaptic triceps e.p.s.p.s in triceps *vs.* non-triceps motoneurones.

The major result was that these sensory–motor connexions had their adult level of specificity as soon as they were formed. For example, at stage XVIII, one stage after connexions begin forming, triceps e.p.s.p.s in heteronymous triceps motoneurones were larger than in two types of unrelated motoneurones (5·4 times bigger

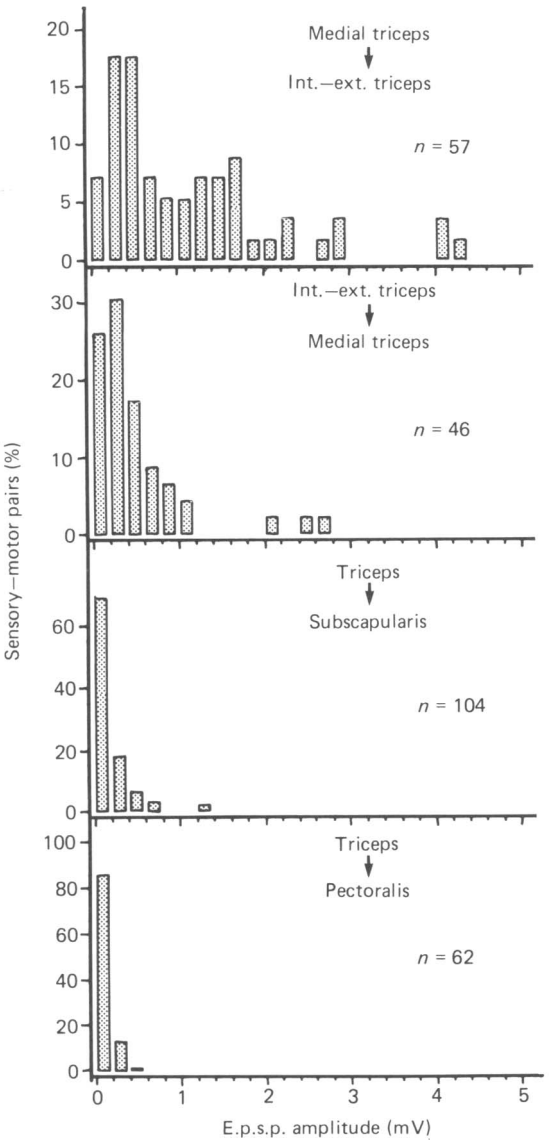

Fig. 5. Amplitude histograms of triceps e.p.s.p.s in four species of brachial motoneurones at stage XVIII. Only those potentials with central latencies less than 3 ms, and therefore probably mediated monosynaptically, are included (see text for details). E.p.s.p.s elicited from triceps sensory afferents are larger in heteronymous triceps motoneurones than in subscapular or pectoralis motoneurones even at this early stage in their formation. Results are from 189 motoneurones in fourteen tadpoles. *n*, number of sensory-motor pairs tested.

than in subscapular motoneurones and 10·5 times bigger than in pectoral motoneurones). These ratios are nearly identical to those in adult animals (Frank & Westerfield, 1982*a*). Amplitude histograms of these synaptic potentials are shown in Fig. 5. Over 64 % of triceps motoneurones have triceps sensory input larger than 0·4 mV, whereas fewer than 10 % of the non-triceps motoneurones have triceps inputs this large.

This pattern of specificity was seen at every developmental stage tested. Triceps e.p.s.p.s were, on average, 5–10 times larger in heteronymous triceps motoneurones than in non-triceps motoneurones. These results are summarized in Fig. 6, which shows a graph of the average triceps sensory input to four different types of brachial motoneurones. Two of the types are triceps motoneurones while the other two are non-triceps motoneurones. At no time during development is there a point where triceps sensory axons provide substantial input to the two types of non-triceps motoneurones we tested.

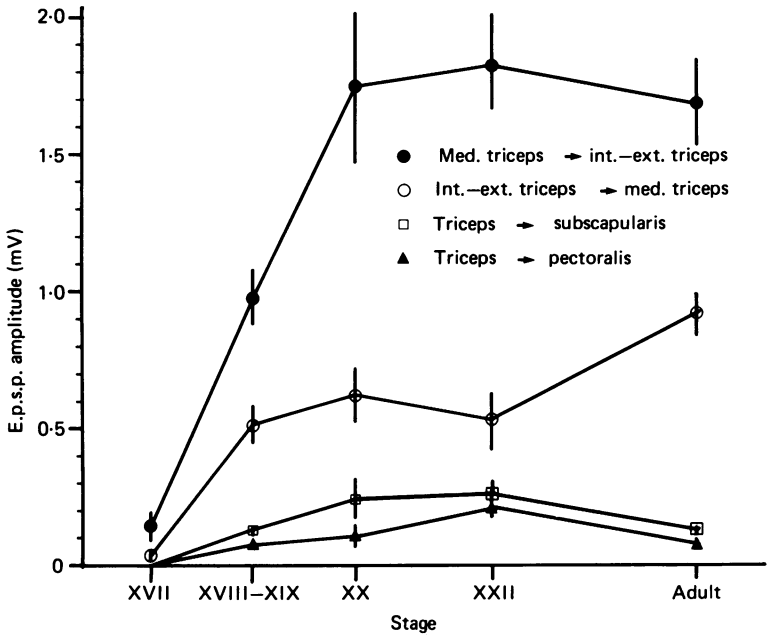

Fig. 6. Average amplitude of monosynaptic triceps e.p.s.p.s (see legend to Fig. 5) in four species of brachial motoneurones at various developmental stages. Vertical bars represent one S.E. of the mean. The preference of triceps sensory axons for triceps motoneurones is apparent at every stage; at no time is there a significant triceps projection to subscapular or pectoralis motoneurones. Results are from 521 motoneurones in forty-eight tadpoles and frogs.

The average amplitudes shown in Fig. 6 could conceal a small population of non-triceps motoneurones that *did* get abnormally large triceps input; these could be cells on which inappropriate connexions are transiently made. However, examination of the individual amplitude histograms for these non-triceps cells (Fig. 7) at several different stages showed no clear evidence for such a population. In each one of the middle histograms, there are one or two cells that received triceps input larger than any seen in the adult sample, but these small numbers are not statistically significant. The results suggest either that no significant number of projection errors is made or that such errors are eliminated sufficiently quickly that they never appear as a significant fraction of the total population.

III. Development of non-triceps monosynaptic sensory–motor connexions

One mechanism that could account for the establishment of specific synapses involves precise and differential timing of synapse formation between different classes of sensory and motor cells (Jacobson, 1978; Macagno, 1978). To determine if timing was a plausible mechanism in the triceps motor system, we compared the developmental stages at which several different classes of sensory–motor connexions were established.

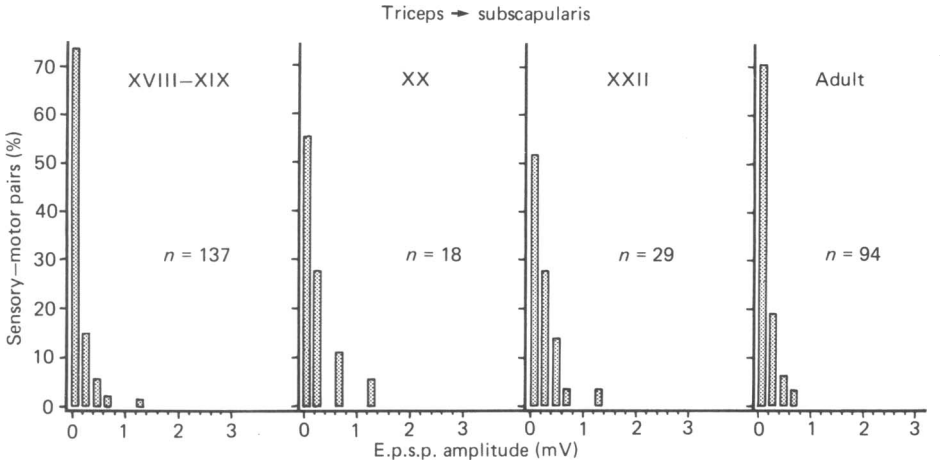

Fig. 7. Amplitude histograms of monosynaptic triceps e.p.s.p.s (see legend to Fig. 5) in subscapular motoneurones at various developmental stages. At no stage are there appreciable numbers of e.p.s.p.s larger than 0·8 mV. *n*, number of sensory–motor pairs tested. Results are from 141 motoneurones in thirty-eight tadpoles and frogs.

The developmental time course of each class of connexions was very similar. No monosynaptic connexion (based on latency measurements) was seen before stage XVII, and the average amplitudes had reached their adult values by stage XX. These results apply to monosynaptic inputs from the triceps, subscapular and pectoral muscle nerves on to triceps, subscapular and pectoral motoneurones. The average amplitudes of e.p.s.p.s for three of these sensory–motor classes are shown in Fig. 8 as a function of developmental stage. We conclude that all types of sensory–motor monosynaptic connexions tested form at about the same time, yet the individual connexions themselves are specific throughout the period of synapse formation.

IV. Mode of synaptic transmission at early sensory–motor synapses

In some systems there is evidence for the formation of electrical connexions between excitable cells that are subsequently replaced by chemical synapses (Fischbach, 1972; MacLeish, 1976). In the adult frog, transmission at sensory–motor synapses is mediated both electrically and chemically (Shapovalov *et al.* 1978; Alvarez-Leefmans *et al.* 1979; Shapovalov & Shiriaev, 1980; Westerfield & Frank, 1982). It was of interest to determine how transmission at these synapses is mediated when they are first formed.

One indication that both chemical and electrical components were present at early

synapses was the presence of a step on the rising phase of some of the triceps e.p.s.p.s. In adult frogs, the first component occurs within a fraction of a millisecond (at 14 °C) of the sensory impulse and is mediated electrically, whereas the second phase occurs with an additional delay of 1–2 ms and is chemically mediated (Shapovalov *et al.* 1978; Alvarez-Leefmans *et al.* 1979; Shapovalov & Shiriaev, 1980; Westerfield & Frank, 1982). Triceps e.p.s.p.s in stage XIX tadpoles showed similar characteristics (Fig. 3). Perfusion with saline containing reduced Ca^{2+} and elevated Mg^{2+} reversibly

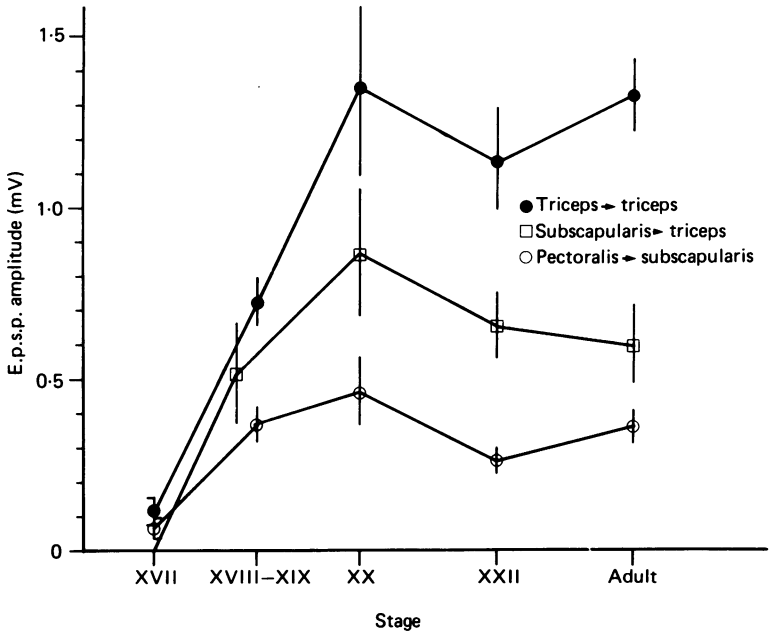

Fig. 8. Average amplitudes of monosynaptic e.p.s.p.s (see legend to Fig. 5) from three classes of muscle afferents in two types of motoneurones at various developmental stages. Both classes of heteronymous triceps connexions (medial triceps sensory axons on to internal–external triceps motoneurones and vice versa) have been combined as a single class. Vertical bars represent one S.E. of the mean. All three synaptic pathways develop during the same time period. Results are from 659 motoneurones in forty-eight tadpoles and frogs.

reduced the second component but not the first, supporting the idea that only the second is mediated chemically.

Extracellular recordings from the central end of the cut ventral root gave a similar result, as illustrated in Fig. 9. In low Ca^{2+} solutions, the monosynaptic component (arrows), corresponding to the chemical component of the e.p.s.p., was reversibly blocked at each stage tested (stages XVII, XIX, and young adult) although the sensory field persisted. This suggests that a major part of the monosynaptic triceps e.p.s.p. is mediated chemically at these stages.

The late, synaptic component of ventral root potentials measured at stages XIII–XV was also reversibly blocked by low Ca^{2+} solutions, consistent with chemical transmission. However, these responses were presumably polysynaptic, and alterations in Ca^{2+} concentration could produce changes in the threshold of interneurones, so the evidence is not as compelling as for monosynaptic pathways.

The sensory field potential was also reversibly reduced in low Ca^{2+} saline, although to a much lesser extent than the synaptic potential. This partial blockade by low Ca^{2+} concentrations suggests that some component of the action potentials in sensory axons within the spinal cord may be carried by, or sensitive to, calcium.

Biphasic intracellular triceps e.p.s.p.s like that shown for stage XIX in Fig. 3 were also seen at stage XVIII (refer to latency histogram in Fig. 2). Taken together, these results show that both forms of synaptic transmission are present in this monosynaptic pathway by stages XVIII and XIX, only one to two stages after the connexions first begin to form.

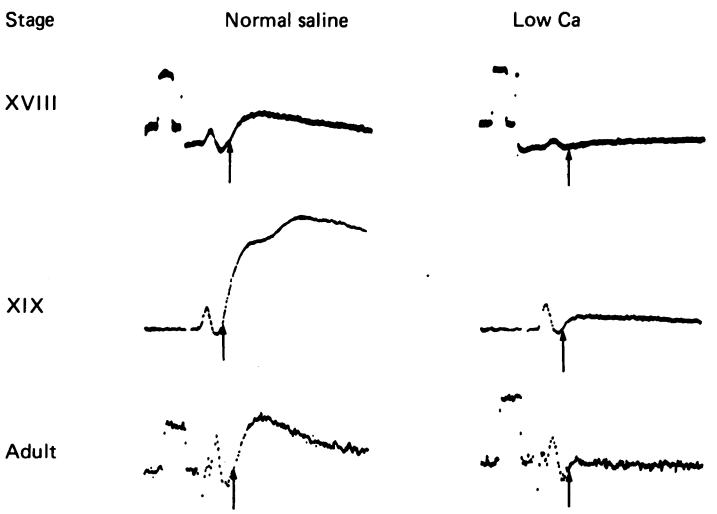

Fig. 9. Effect of low Ca^{2+}, high Mg^{2+} saline on triceps e.p.s.p.s in motoneurones recorded extracellularly in the ventral root. Low Ca^{2+} saline contained 0·3 mM-Ca^{2+}, 9 mM-Mg^{2+} for Stages XVIII and adult, and 0·5 mM-Ca^{2+}, 7·5 mM-Mg^{2+} for Stage XIX. The monosynaptic chemical components (arrows) are either blocked (stages XVIII and adult) or greatly reduced (stage XIX) in low Ca^{2+} saline. The traces shown for normal saline at stage XIX were recorded 75 min *after* those in low Ca^{2+}, during the rinse. Calibration, 2 ms. Each trace is the average of three to ten individual responses.

DISCUSSION

First appearance of monosynaptic sensory–motor connexions

Monosynaptic connexions between triceps brachii muscle sensory and motor neurones first appear relatively late during larval development in bullfrogs, well after functional innervation of muscle is established. Letinsky (1974) found that motor axons are present in the muscle nerves by stages VI–VII and neuromuscular synapses are functional in hind limb muscles by stages VII–VIII. Reflex activity can be evoked by stages IX–X. We tested for functional central connexions as early as stage XIV and recorded a few long-latency, presumably polysynaptic responses. Not until stage XVII, however, was there evidence of a monosynaptic pathway between sensory and motor cells. This is only one or two stages before metamorphic climax (stage XX), when the forelimbs erupt through the body wall and the tadpole begins to use his forelimbs to move around. Sensory–motor e.p.s.p.s attain their adult amplitudes in

only one or two weeks, compared to a total larval development of at least three months.

In mammals, the stretch reflex is present at birth (Skogland, 1960*a*,*b*). Eccles *et al.* (1963) showed that in kittens from 19 to 45 days of age, monosynaptic connexions were present between low threshold muscle sensory axons (presumably from muscle spindles) and lumbar motoneurones. Naka (1964*a*,*b*) demonstrated that these connexions were present even one to two weeks before birth, but no one has described when they first appear. Recordings made from isolated spinal cords of fetal rats have shown that short latency potentials can be recorded in the ventral roots in response to dorsal root stimulation beginning on embryonic day 17·5 (Saito, 1979). This agrees well with recent anatomical findings (Carolyn Smith, personal communication) showing that anatomical contacts between sensory and motor cells innervating intercostal muscles in the rat first appear around embryonic day 18. Thus, as in the frog, the stretch reflex appears to develop only shortly before the animal uses his limbs and breathes.

Specificity of newly formed sensory–motor synapses

The projection of triceps muscle sensory neurones on to various classes of brachial motoneurones was remarkably specific from the time that these monosynaptic connexions first appeared. This specificity was measured by comparing the ratio of triceps sensory e.p.s.p.s in triceps *vs.* non-triceps motoneurones. Triceps sensory cells always produced larger monosynaptic e.p.s.p.s in triceps motoneurones than in either pectoralis or subscapularis motoneurones. Throughout the development of this monosynaptic pathway, the specificity of these connexions is approximately the same as in the mature animal.

An obvious interpretation of these observations is that triceps sensory axons never make appreciable numbers of errors as they innervate brachial motoneurones. However, inappropriate connexions might be transiently formed and then quickly corrected. Synaptogenesis in this system probably occurs over a period of one to several weeks. If each inappropriate connexion were corrected (i.e. broken) shortly after its formation, the cumulative number of errors present at any one point in time would remain small and might be undetectable with our sampling methods. Although we found no positive evidence favouring this second hypothesis, it is consistent with our observations. We raise it as a possibility because it would allow for some types of error correction that might be important in determining synaptic specificity.

In the peripheral nervous system, synaptic connexions undergo a major rearrangement during development (for a review, see Purves & Lichtman, 1980). Initially, each presynaptic axon innervates a large number of post-synaptic target cells. The input to many of these targets is then lost, leaving each presynaptic axon innervating a greatly reduced number of target cells. This phenomenon is called synapse elimination, even though the total number of synaptic contacts increases during the time that the multiple projections are being reduced. Muscle sensory axons might similarly initially innervate many different types of motoneurones and then eliminate their contacts with all but the functionally appropriate ones (Saito, 1979). In the sensory–motor system we have studied, however, there is no time when the pattern of connexions is appreciably different than in the adult. Synaptic specificity here is apparently not increased by selective synapse elimination.

In those systems where synapse elimination has been studied most thoroughly, the synaptic specificity of the system is also not dramatically increased by elimination of 'inappropriate' synapses. Initially a motoneurone projects only to the correct muscle (Landmesser, 1980). Similarly, post-ganglionic cells in the superior cervical ganglion are initially innervated by axons from contiguous spinal roots, just as in adults (Lichtman & Purves, 1980). During synapse elimination, the terminals of each presynaptic axon are rearranged so that it innervates a much smaller fraction of the total number of 'appropriate' targets. The analogous process for the sensory–motor system would be for a muscle sensory neurone to lose its connexions not with 'inappropriate' motoneurones, but with 'appropriate' ones. Since single muscle afferent axons innervate over 90% of homonymous motoneurones in adult animals (Mendell & Henneman, 1971; Lichtman & Frank, 1981), there is simply not much room for the type of synapse elimination that is observed in the peripheral nervous system. The synaptic contacts between individual sensory and motor neurones might undergo considerable rearrangements during development, however. To determine if such changes occur, it will be necessary to study the anatomical and physiological projections of individual sensory axons.

Reduction of polysynaptic inputs

In contrast to the unchanging pattern of monosynaptic inputs from muscle sensory afferents to motoneurones, longer-latency polysynaptic e.p.s.p.s become less prominent as development proceeds. This was true both for responses recorded intracellularly, and for extracellular recordings from the ventral root (refer to Fig. 4). A similar reduction of longer-latency inputs is evident in ventral root recordings from fetal and neonatal spinal cords of the rat (Saito, 1979).

One interpretation of these results, suggested by Saito, is that this reflects a process of synapse elimination. For example, if muscle sensory axons lost their inputs to certain interneurones, then the polysynaptic sensory–motor potentials would be reduced. However, we do not view our results as good evidence even for synapse elimination in polysynaptic pathways, since there are alternate interpretations. For example, interneurones grow in size and presumably in input conductance as development proceeds. The same synaptic input sufficient to bring a small interneurone to threshold might remain subthreshold for activation of a larger one. Thus the polysynaptic response could disappear during development without elimination of synaptic inputs.

Comparison with other developmental studies of the sensory–motor pathway

The monosynaptic connexions between muscle sensory axons and lumbar motoneurones were studied in young kittens by Eccles and her collaborators (Eccles *et al.* 1963; Eccles & Willis, 1965). In agreement with the observations reported here, they found that the basic pattern of the connexions was like that in adult cats. However, two types of motoneurone *did* receive significantly greater monosynaptic inputs from particular muscle nerves than in adults. This loss of input during development could be explained by synapse elimination. An alternative explanation is based on their observation that e.p.s.p. amplitudes were often 1·5–2 times larger than in adults, a phenomenon we did not observe in frogs. As motoneurones grow, their input impedance may fall; the same amount of synaptic input would then produce a smaller

e.p.s.p. as suggested by Eccles & Willis (1965). This would facilitate the detection of e.p.s.p.s in kittens that have small amplitudes in adults, and might reveal some 'aberrant' connexions that were simply undetectable in adults. It should be mentioned, however, that Eccles and her collaborators looked at over thirty types of sensory–motor pairs, finding aberrant neonatal connexions in only two types, whereas we have studied only six to eight different types in detail. We may find some rearrangements in other classes of connexions.

Saito (1979) also studied the development of sensory–motor connexions in the spinal cords of rats. He stimulated dorsal roots while recording the responses in motoneurones extracellularly in the ventral root L3. Soon after short-latency responses appeared (embryonic day 17·5), reflex discharges in L3 were evoked by stimulating any dorsal root from L1 to L5. After birth, however, these responses could be obtained only when L3 or L4 dorsal roots were stimulated. Saito suggested that synapse elimination might be responsible for the loss of the intersegmental reflex. This work is difficult to compare directly with our own, since both muscle and cutaneous sensory fibres were presumably stimulated in the dorsal roots. The slow time base used in his figures of ventral root recordings makes it difficult to distinguish monosynaptic muscle sensory input from strong but polysynaptic cutaneous input, which in the frog has a minimal latency only two or three milliseconds longer than the monosynaptic input (E. Frank, unpublished observations). If the intersegmental reflex described by Saito in fetal animals does represent a polysynaptic pathway, its elimination might be analogous to the loss of polysynaptic inputs we observe in the frog. Its disappearance would then not require the selective loss of synaptic connexions.

Possible mechanisms of synaptic specificity

Two theories that have been proposed to explain the development of specific synaptic connexions are based on precise spatial relationships or timing of synaptogenesis. A timing hypothesis appears to be inadequate to explain our results, as already discussed. Wyman, however, has proposed that the pattern of monosynaptic sensory–motor connexions in the cat could be explained largely on the basis of the anatomical position of different species of motoneurones (Wyman, 1973). This mechanism could not explain the specificity we see in the frog since all the synapses we have studied are located in the same region of spinal cord. All three types of motoneurones (triceps, subscapularis, and pectoralis) are located in overlapping rostrocaudal positions, and labelling of these neurones with horseradish peroxidase shows that their dendrites occupy the same space (J. W. Lichtman, S. Jhaveri & E. Frank, in preparation). Thus the basis of this specificity cannot simply be for a sensory axon to project to the appropriate region of the spinal cord. A similar conclusion can be made concerning the selective innervation of post-ganglionic cells in the superior cervical ganglion (Lichtman, Purves & Yip, 1979). Instead, sensory axons must be able to recognize, in a nearly all-or-none manner, appropriate motoneurones even though their dendrites are surrounded by dendrites of inappropriate motoneurones. This remarkable specificity is apparent from the moment that sensory–motor synapses become functional.

Our observations on the development of this reflex pathway can be well explained by mechanisms involving the chemical recognition between appropriate classes of

sensory and motor cells, such as the chemospecificity hypothesis originally proposed by Sperry (1963). Neurones might become chemically specified before reaching their peripheral targets, as seems to be the case for the projection of motoneurones to muscles (see Landmesser, 1980, for review) and for preganglionic sympathetic axons to peripheral ganglia (Lichtman & Purves, 1980). Alternatively, the specification might be the *result* of innervating a particular peripheral target. This second possibility has received most attention in relation to the central connexions of sensory neurones (Miner, 1956; Baker & Jacobson, 1970; Frank & Westerfield, 1982*b*). The chemical recognition between sensory and motor neurones might occur at the moment of contact, before functional synapses have formed, or shortly after synapse formation. In either case the pattern of synaptic connexions would appear to be correct from the very beginning.

An alternative scheme involves the correlation of neuronal electrical activity in pre- and post-synaptic cells. This hypothesis provides an attractive explanation for the development of ocular dominance columns in the visual cortex of mammals (Stryker, 1981). In the present context, muscle stretch receptor axons become active just after contraction of the muscle they innervate (Ito, 1968); a burst of impulses in motoneurones followed by a burst in the sensory neurones might reinforce homonymous and synergistic sensory–motor connexions. A potential disadvantage of this hypothesis is that muscle afferent axons are strongly activated by contraction of antagonistic muscles, yet antagonistic sensory–motor e.p.s.p.s are rarely seen. It will be interesting to see if specific sensory–motor connexions will develop in an animal whose limb is paralysed with tetrodotoxin or curare.

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