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DEVELOPMENT OF THE MONOSYNAPTIC STRETCH REFLEX IN THE RAT: AN *IN VITRO* STUDY

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SUMMARY

1. The properties and development of the stretch reflex pathway were investigated in new-born and fetal rats using the isolated spinal cord–hind limb preparation.

2. Muscle afferent discharge was elicited by small stretch of the triceps surae muscle in the new-born rat and in the fetus. It appeared as early as embryonic day 18·5. Ramp-and-hold stretch elicited only phasic discharges in most afferent fibres.

3. A phasic reflex response was evoked in the triceps surae muscle by brief or ramp-and-hold stretch of the muscle in the new-born rat. The threshold stretch required for evoking the reflex response was close to that for eliciting the afferent discharge.

4. A reflex response in the triceps surae muscle was also evoked by electrical stimulation of the triceps surae muscle nerve or the sciatic nerve in the new-born rat.

5. Excitatory post-synaptic potentials (e.p.s.p.s) in the triceps surae motoneurones were evoked by stimulation of the muscle nerve in the new-born rat. The amplitude of the e.p.s.p.s was large enough to generate spike potentials. Homonymous e.p.s.p.s were significantly larger than heteronymous e.p.s.p.s.

6. The amplitudes of the e.p.s.p.s were very susceptible to the rate of stimulus repetition. At a stimulus frequency of 10 Hz they were depressed to less than 10% of the control value.

7. Presynaptic impulses evoked by stimulation of afferents in the muscle nerve appear in the motor nucleus less than 1·0 ms before the onset of synaptically evoked field potentials. The interval between the arrival of impulses evoked by dorsal root stimulation and the onset of e.p.s.p.s in motoneurones was $0{\cdot}56 \pm 0{\cdot}16$ ms, indicating monosynaptic transmission from the primary afferents to the motoneurones.

8. In the fetus, a reflex response in the triceps surae muscle was observed following a small stretch of the muscle (or electrical stimulation of the sciatic nerve) in all preparations at embryonic day 20·5 and in about half of those examined at embryonic day 19·5. Neither stimulation evoked a reflex response at embryonic day 18·5. Latencies of the reflex responses evoked by muscle stretch or by nerve stimulation were similar to those in the new-born rat. It is concluded that the monosynaptically evoked stretch reflex response in the triceps surae muscle first appear at embryonic day 19·5.

9. Natural and electrical stimulation of the plantar skin evoked a reflex response with long latencies in flexor muscles. Such a cutaneous reflex was first present at embryonic day 17·5, two days earlier than the onset of the stretch reflex.

INTRODUCTION

Stretching a muscle induces contraction via the monosynaptic reflex pathway. In the mammal, the post-natal development of the monosynaptic pathway has been investigated electrophysiologically in the cat (Skoglund, 1960*a*, *b*, *d*; Wilson, 1962; Eccles, Shealy & Willis, 1963; Eccles & Willis, 1965; Kellerth, Mellström & Skoglund, 1971; Mellström, 1971). These studies have shown that in the lumbar spinal cord the pathway is already functional at birth. In fact, Naka (1964) recorded excitatory post-synaptic potentials (e.p.s.p.s), probably monosynaptically evoked, in motoneurones after stimulation of dorsal root or peripheral nerve fibres in the kitten fetus.

Systematic studies of the prenatal development of spinal reflexes have been performed in the rat fetus, in which the gestation age can be determined precisely (Angulo, 1932; Windle & Baxter, 1936; Narayanan, Fox & Hamburger, 1971; May & Biscoe, 1975; Saito, 1979). Behavioural observations have revealed that reflex activities in the hind limb region first appear at embryonic day 17, four days before birth (Narayanan *et al.* 1971). Segmental reflex responses were detected even earlier (embryonic day 15·5) by recording ventral root discharges during an *in vitro* study of the rat fetus (Saito, 1979). In those experiments, however, stimulation was applied to the skin or the dorsal root. Spinal reflexes originating from muscles have not been studied systematically in the fetus, so it is not known at what stage of development monosynaptic reflexes can first be evoked by volleys in muscle afferent fibres; nor have the properties of the reflex pathway been determined for the early stages of development.

The present study was, therefore, undertaken to examine the development of the monosynaptic stretch reflex pathway in new-born and fetal rats using the isolated spinal cord–hind limb preparation. It will be shown that the monosynaptic stretch reflex loops are formed before birth. The reflex response in the ankle extensor muscle first appears at embryonic day 19·5, two days later than the polysynaptically evoked cutaneous reflex in flexor muscles. Properties of the afferent discharges elicited by muscle stretch in new-born and fetal rats are also described.

METHODS

Twenty-two new-born and sixty fetal rats of the Wistar strain were used. Ages in days of post-natal rats were numbered from birth. Fetal ages were calculated from the day on which sperm were found in the vaginal smear of the mother. This day was designated embryonic day 0·5. The crown–rump length of the fetuses was measured at the beginning of each experiment to countercheck the age of the litter (Narayanan *et al.* 1971). The gestation period was usually 21·5 days. Ages of rats in the present study ranged between embryonic day 16·5 and post-natal day 3.

Preparations

The new-born rat was decapitated and eviscerated under ether general anaesthesia. Laminectomy and dissection of the left hind limb nerves were performed in Krebs solution bubbled with 95% O_2 and 5% CO_2. The spinal cord caudal to the thoracic level was split mid-sagittally. The left half of the spinal cord, the 4th and 5th lumbar (L4 and L5) spinal roots (both dorsal and ventral roots kept intact), the sciatic nerve and the triceps surae muscle were isolated *en bloc*. Small parts of the femur and the tibia to which the proximal tendon of the triceps surae muscle was attached were sometimes isolated together to facilitate examination of responses to stretch. In some cases, the innervation of the biceps femoris muscle and the foot were also kept intact.

The fetuses were removed by Caesarean section from rats fully anaesthetized with ether. The fetus was decapitated and eviscerated in a dissection chamber filled with Krebs solution. By removing the ventral bodies from the thoracic to the sacral level the ventral surface of the spinal cord was exposed to keep the preparation in good condition. In the left hind limb, the biceps femoris muscle was removed and the sciatic nerve was separated from the surrounding tissue. The innervation of the triceps surae muscle was kept intact. The distal portion of the triceps surae muscle and the calcaneus and neighbouring bones attached to the distal tendon were freed from surrounding tissues to allow controlled stretching of the muscle. In the right hind limb, the biceps femoris muscle was exposed for electromyographic recording. In some cases ankle flexor muscles were also exposed.

The isolated preparation was placed in an experimental chamber perfused steadily (1–2 ml/min) with Krebs solution (118·4 mM-NaCl, 4·69 mM-KCl, 2·52 mM-$CaCl_2$, 1·25 mM-$MgSO_4$, 25·0 mM-$NaHCO_3$, 1·18 mM-KH_2PO_4, 11·1 mM-glucose) saturated with 95% O_2 and 5% CO_2. In some experiments $CaCl_2$ in Krebs solution was replaced with 5 mM-$MgSO_4$. In new-born rats, the spinal cord (with its medial cut surface upward) and the bones to which the proximal tendon of the triceps surae muscle was attached were fixed firmly on the silicone base of the chamber with insect pins. The preparations of fetuses were fixed by anchoring the femur and the tibia on the base of the chamber. The temperature of the bathing solution was maintained at 25 ± 1 °C. The spinal reflexes as described below could be observed for periods longer than 10 h.

Stimulation

The cut ends of pairs of insulated silver wire electrodes (diameter, 100 μm) were placed on the triceps surae muscle nerve, on the sciatic nerve or on the plantar skin. In fifteen experiments on new-born rats, the triceps surae muscle nerve was further divided into the medial gastrocnemius and lateral gastrocnemius-soleus nerves and stimulation electrodes were placed on each of them. The nerve was stimulated with a pulse of 0·2 ms duration.

For stretching the triceps surae muscle the Achilles tendon (new-born rats) or the bones attached to it (fetuses) were tied with thin silk thread and connected to the electro-magnetic stretcher (ME Commercial). The initial length of the triceps surae muscle was set at a length comparable to that when the ankle joint was angled at 90–100 deg (mean initial length; 3·8 mm at embryonic day 18·5, 4·8 mm at embryonic day 19·5, 6·2 mm at embryonic day 20·5 and 7·3 mm at 0-day-old). Brief or ramp-and-hold stretches of the muscle of up to 400 μm were produced. The rise time for tension production during stretch was 10–20 ms. Displacement amplitude could be measured to an accuracy of 10 μm.

Recording

The electromyogram (e.m.g.) of triceps surae, biceps femoris or ankle flexor muscles was recorded with pairs of platinum–iridium wires (20 μm diameter, insulated except for the tip) placed on the surface of the muscle belly. Intracellular recording from motoneurones was performed only in new-born rats. Glass micropipettes filled with 4 M-potassium acetate were used. Resistance, measured by direct current, was initially 40–60 MΩ, but often increased up to about 100 MΩ when used for recording. Motoneurones were identified via the antidromic action potentials evoked by stimulation of medial gastrocnemius or lateral gastrocnemius-soleus nerves, and in some cases by stimulation of the L4 or L5 ventral root. For recording field potentials in the spinal cord, glass micropipettes filled with 2 M-sodium chloride (resistance, 1–2 MΩ) were used. Afferent discharges were recorded monopolarly with a silver wire electrode placed under the dorsal root (new-born rats) or the sciatic nerve (fetuses) in the air or in liquid paraffin. The indifferent electrode was a silver–silver chloride plate placed in the solution in the chamber. The recorded field potentials were averaged in an averaging computer (ATAC 350, Nihon Kohden) to increase the signal to noise ratio (Fig. 5).

RESULTS

Properties of the spinal reflex evoked by afferent volleys in muscle nerves in the new-born rat

The reflex response evoked by stretching the triceps surae muscle, or by electrically stimulating the nerve to it, was examined by recording the e.m.g. or by intracellular recording from motoneurones in 0–3-day-old rats.

Effect of muscle stretch

Afferent discharge. The effect of stretch of the triceps surae muscle was examined by recording from the dorsal root in four 0-day-old rats. Fig. 1 shows sample records of afferent discharges in the dorsal root of the 5th lumbar segment (L5). Only phasic discharges were elicited by a small ramp-and-hold stretch. As stretch of the muscle

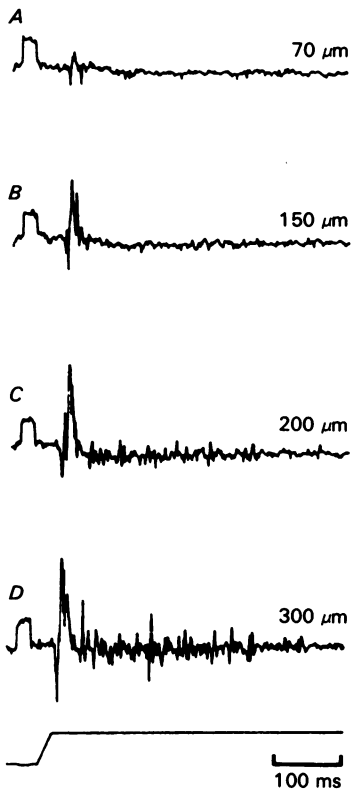

Fig. 1. Afferent discharges evoked by ramp-and-hold stretch of the triceps surae muscle. A 0-day-old rat. Discharges were recorded from the L5 dorsal root following stretches of 70 (*A*), 150 (*B*), 200 (*C*) and 300 μm (*D*). Negativity upward. The lowest trace shows the muscle displacement. Calibration pulse: 100 μV.

was intensified, the amplitude of the phasic discharges increased gradually as shown in *A–D*. Tonic discharges became conspicuous when stretch was more than 200 μm (*C* and *D*). The threshold stretches for evoking phasic and tonic discharges were 50–100 μm and 150–200 μm, respectively. The latter threshold was usually 2–3 times higher than the former in any one preparation.

Reflex response. Brief stretch (duration, 60 ms) or ramp-and-hold stretch of a triceps surae muscle evoked a phasic e.m.g. response in the stetched muscle in all of the seven 0-day-old rats examined. The muscle response was of spinal reflex origin, since it disappeared after severance of the dorsal root. Fig. 2*A* represents the e.m.g. recording from a triceps surae muscle following brief stretches of various amplitudes

in a 0-day-old rat. The phasic e.m.g. response appears at a stretch of 70 μm and becomes maximal at a stretch of 150 μm. Latencies for the maximum response, measured from the onset of stretch, were 56·3–84·5 ms (mean, 69·7 ms; $n = 7$) and thresholds of stretch for evoking the muscle response were 50–120 μm.

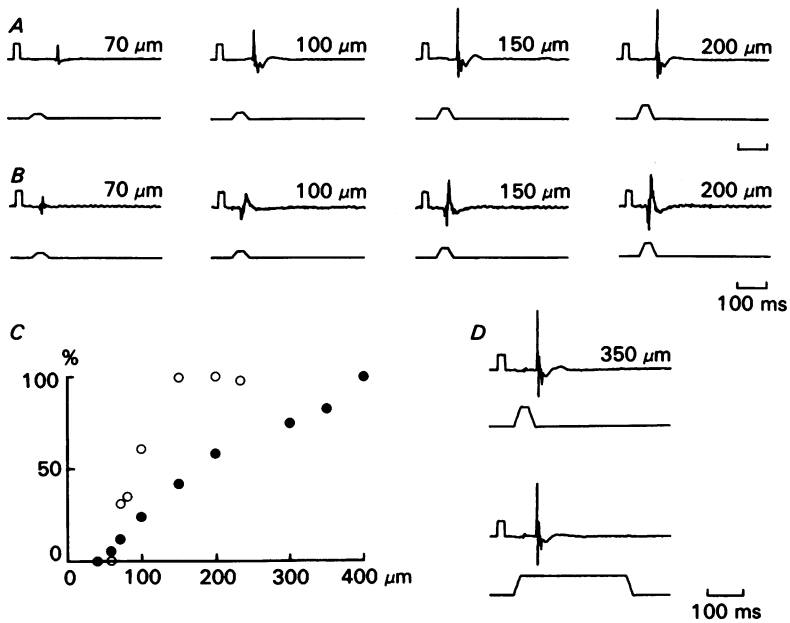

Fig. 2. Reflex responses in the triceps surae muscle (*A* and *D*) and afferent discharges in the dorsal root (*B*) evoked by muscle stretch. *A–C*, a 0-day-old rat. In *A*, upper traces show e.m.g. recordings from the triceps surae muscle following brief stretches of the muscle (70, 100, 150 and 200 μm). Lower traces, displacements. In *B*, upper traces show afferent discharges following stretch of the triceps surae muscle (70, 100, 150 and 200 μm), recorded from the peripheral end of the cut L5 dorsal root. Lower traces, displacements. Calibration pulse: 500 μV (*A*) and 100 μV (*B*). *C*, amplitudes of reflex responses (open circles) and afferent discharges (filled circles) plotted as a function of the amplitude of stretching. The largest reflex responses and afferent discharges were taken as 100%. *D*, reflex e.m.g. responses in the triceps surae muscle evoked by brief (upper record) and ramp-and-hold (lower record) stretch of the muscle in another 0-day-old rat. The amplitude of stretch was 350 μm. Calibration pulse: 500 μV.

As shown in Fig. 2*D* there was no difference between the responses evoked by brief stretches or by ramp-and-hold stretches with the same rise time and amplitude. Tonic responses did not appear during maintained stretch in any of the animals examined, even when stretch amplitude was large.

The reflex e.m.g. responses (Fig. 2*A*) and the afferent nerve discharges (Fig. 2*B*) evoked by stretches of various amplitudes were recorded in the same preparations. In Fig. 2*C*, the amplitudes of the reflex responses (open circles) and those of the afferent discharges (filled circles) are plotted against the amplitude of stretch. Note that activity in small populations of afferent fibres is sufficient to elicit a reflex response. The maximum response is obtained following stretch of 150 μm, whereas

the afferent discharges are still submaximal at 400 μm (the largest stretch in this study). Similar observations were made in another two 0-day-old rats. The minimum amplitude of stretch for evoking a reflex response was slightly larger than that for evoking an afferent discharge, but the difference was less than 20 μm in each case examined.

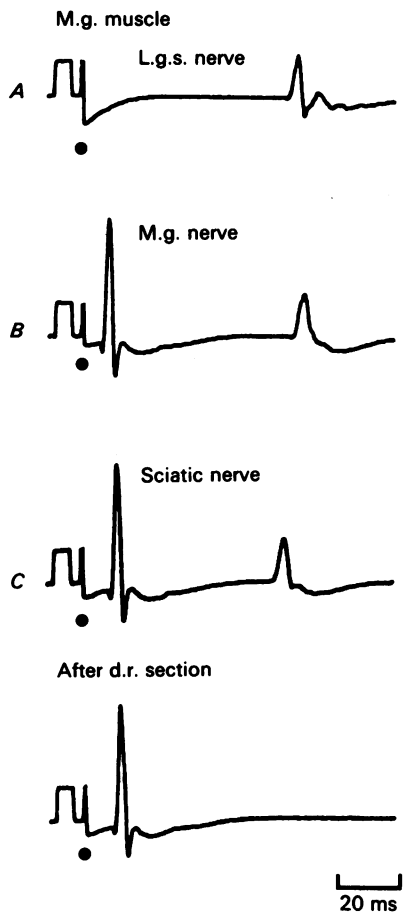

Fig. 3. Reflex responses evoked by peripheral nerve stimulation. E.m.g. recordings from the medial gastrocnemius (m.g.) muscle in a 1-day-old rat. Stimulation was applied to the lateral gastrocnemius-soleus nerve (l.g.s. nerve; *A*), the m.g. nerve (*B*) and the sciatic nerve (*C*) at 0·1 Hz. Lower trace in *C*, the response evoked by sciatic nerve stimulation after section of the dorsal roots (d.r.). Calibration pulse: 500 μV. Dots indicate stimulus artifacts.

Effect of muscle nerve stimulation

Reflex e.m.g. response. Fig. 3 represents e.m.g. responses in the medial gastrocnemius muscle after supramaximal stimulation of various peripheral nerves in a 1-day-old rat. Stimulation of the medial gastrocnemius nerve evoked two components of muscle response with latencies of 5·1 and 63·1 ms (*B*), while stimulation of the lateral gastrocnemius-soleus nerve evoked only the later response (*A*). When the homonymous muscle nerve was stimulated, the threshold for the early muscle response was

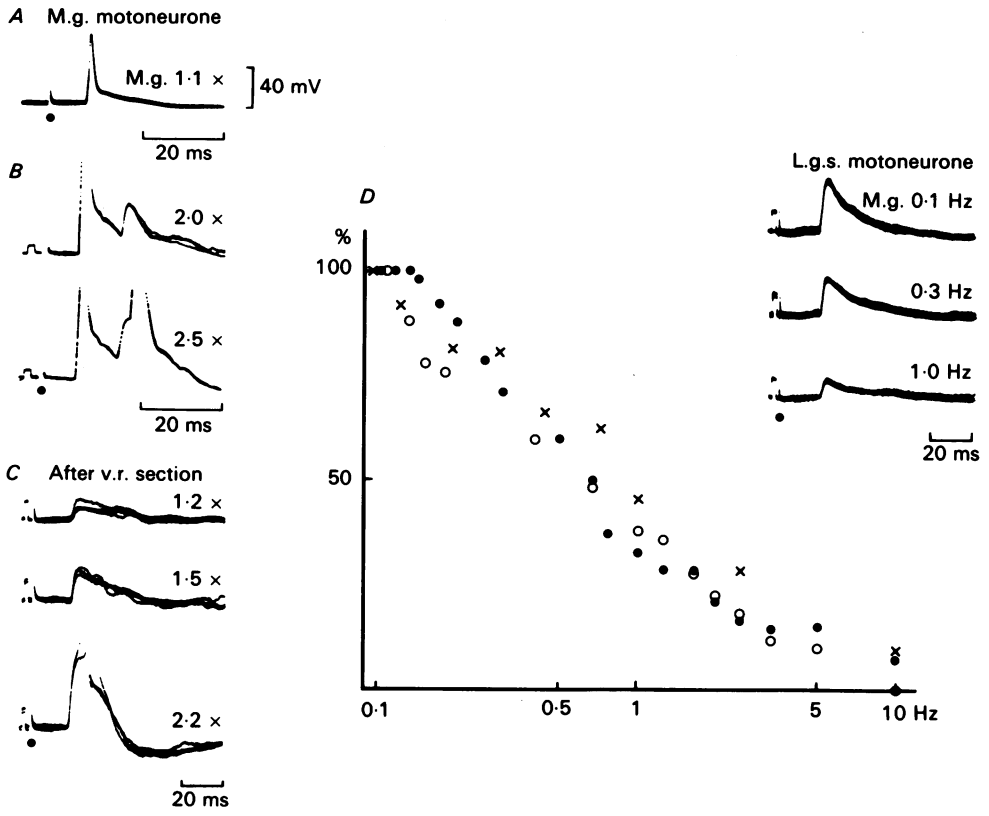

Fig. 4. Effects of muscle nerve stimulation on motoneurones. *A–C*, intracellular recordings from a medial gastrocnemius (m.g.) motoneurone in a 2-day-old rat. *A*, antidromic action potential evoked by m.g. nerve stimulation just above the threshold. *B*, antidromic action potentials and succeeding e.p.s.p.s evoked by m.g. nerve stimulation. Stimulus intensities are 2·0 (upper) and 2·5 (lower) times the threshold for antidromic activation. *C*, effects of m.g. nerve stimulation on the same motoneurone after section of the ventral root (v.r.). Stimulus intensities are 1·2, 1·5 and 2·2 times the threshold for antidromic activation in the top, middle and bottom trace, respectively. Note that *A* and *B* are faster sweep than *C*. Calibration pulse: 2 mV (*B* and *C*). *D*, effects of frequency of stimulation on e.p.s.p.s. Inset records, e.p.s.p.s in a lateral gastrocnemius-soleus (l.g.s.) motoneurone evoked by m.g. nerve stimulation at 0·1 (top), 0·3 (middle) and 1·0 Hz (bottom). The relation between amplitudes of e.p.s.p.s (ordinate) and frequency of stimulation (abscissa) for heteronymous e.p.s.p.s in two l.g.s. motoneurones (filled and open circles) of a 2-day-old rat. Crosses obtained from e.p.s.p.s following dorsal root stimulation in a motoneurone of a 0-day-old rat. Dots indicate stimulus artifacts.

lower than for the late response. As stimulus intensity was increased, the late response appeared and augmented. Usually the largest late responses (10–30 % of the maximum amplitude of the early response) were evoked by supramaximal stimulation of the nerve. Two components of muscle response were also evoked by stimulation of the sciatic nerve. The latency of the early response was then longer, and that of the late response was shorter than when the homonymous muscle nerve was stimulated (upper trace in *C*, compared with *B*). The early response was produced directly by volleys in the motor nerve and the late one was of spinal reflex origin, since only the latter

disappeared after the dorsal root was cut (lower trace in *C*). Reflex responses were observed following sciatic nerve stimulation in all five 0–1-day-old rats.

Excitatory post-synaptic potentials (*e.p.s.p.s*). For further analysis of the reflex effects of muscle nerve stimulation, intracellular recordings were made from motoneurones innervating the triceps surae muscle in fifteen new-born rats. Though the majority of motoneurones were injured by insertion of the micro-electrode and deteriorated in several minutes, the effect of stimulation of lateral gastrocnemius-soleus or medial gastrocnemius nerves could be well examined in twenty-one lateral gastrocnemius-soleus and nine medial gastrocnemius motoneurones with resting membrane potentials in excess of 40 mV.

Synaptic potentials from muscle afferents were not detectable in motoneurones when the homonymous muscle nerve was stimulated at or above the threshold intensity for antidromic activation, because the antidromic action potentials usually prevented observation of synaptic potentials. In two motoneurones, however, e.p.s.p.s could be observed on the after-depolarization of the antidromic action potential. Fig. 4*A–C* shows intracellular recordings from an antidromically identified (*A*) medial gastrocnemius motoneurone. Stronger stimuli evoke e.p.s.p.s and spike potentials about 10 ms after the onset of the antidromic activation (*B*). Pure e.p.s.p.s could be observed in the same motoneurone after the ventral root was cut during intracellular recordings from the motoneurone as shown in Fig. 4*C*. As the stimulus intensity increased, the e.p.s.p.s with a fixed latency increased in amplitude and generated a spike potential. A similar observation was obtained in another medial gastrocnemius motoneurone. The amplitude of e.p.s.p.s measured at the threshold intensity for generating spike potentials (after severance of the ventral root) was 8·0 and 8·4 mV in the two motoneurones.

Homonymous e.p.s.p.s were also observed in all the five motoneurones in which the antidromic action potential could be blocked by preceding spike potentials activated directly by intracellular application of a short depolarizing pulse. The amplitudes of e.p.s.p.s thus observed ranged between 2·8 and 6·0 mV (mean $\pm$S.D., 4·9$\pm$1·2 mV). The smaller amplitudes of the homonymous e.p.s.p.s as compared with those obtained after severance of the ventral root could be due to the increased conductance of the motoneurone membrane which follows the direct spike potential (Coombs, Eccles & Fatt, 1955).

Latencies of homonymous e.p.s.p.s were 16·5–24·6 ms (21·0$\pm$3·3 ms) in the seven motoneurones. In individual motoneurones, they were 4·7–9·9 ms (7·6$\pm$2·0 ms) longer than those of antidromic action potentials.

Heteronymous e.p.s.p.s as exemplified in the inset records in Fig. 4*D* were observed in all the thirty motoneurones (medial gastrocnemius and lateral gastrocnemius-soleus) examined. Their latencies ranged between 16·5 and 23·6 ms (20·3$\pm$2·3 ms). The maximum amplitudes of heteronymous e.p.s.p.s were measured in four medial gastrocnemius and thirteen lateral gastrocnemius-soleus motoneurones and found to be 1·0–4·9 mV (2·8$\pm$1·2 mV). They were significantly less than those of homonymous e.p.s.p.s (the Mann–Whitney *U* test, $P < 0{\cdot}02$).

Effect of the repetition rate on amplitudes of e.p.s.p.s. The amplitudes of the e.p.s.p.s described above were measured at repetition rates of 0·3–0·4 Hz. But amplitude depended markedly on the repetition rate. The inset records in Fig. 4*D* show e.p.s.p.s in a lateral gastrocnemius-soleus motoneurone evoked by stimulation of the medial

gastrocnemius nerve at three different frequencies, 0·1 Hz (top), 0·3 Hz (middle) and 1·0 Hz (bottom). The amplitudes of heteronymous e.p.s.p.s in two lateral gastrocnemius-soleus motoneurones of a 2-day-old rat are plotted against the frequency of stimulation in Fig. 4*D* (filled and open circles). The amplitudes were depressed to about half of the control value at a stimulus frequency of as low as 0·6 Hz. Only a trace of e.p.s.p. was observed at 10 Hz. This was also the case for e.p.s.p.s evoked by dorsal root stimulation in a motoneurone of a 0-day-old rat (crosses in Fig. 4*D*).

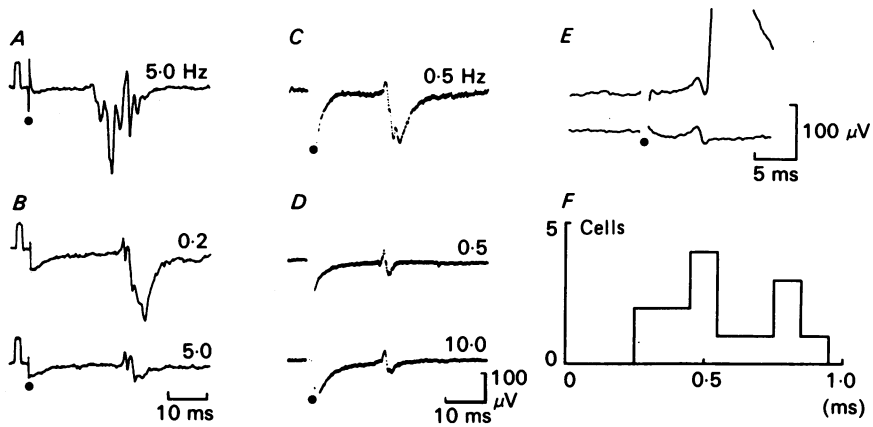

Fig. 5. Presynaptic volleys and trans-synaptic potentials in the motor nucleus. *A* and *B*, field potentials evoked in the motor nucleus by m.g. nerve stimulation in a 1-day-old rat. *A*, both the ventral and the dorsal root of 5th lumbar segment (L5) kept intact. Field potentials evoked by m.g. nerve stimulation at 5·0 Hz. After recording *A*, the L5 ventral root was cut. The recording micro-electrode was located at the same site during this procedure. *B*, field potentials evoked by m.g. nerve stimulation at 0·2 Hz (upper trace) and 5·0 Hz (lower trace) after section of the ventral root. All records were averaged (100 trials). Calibration pulse; 100 μV. *C* and *D*, orthodromic field potentials evoked by l.g.s. nerve stimulation in the l.g.s. motor nucleus in a 3-day-old rat. The ventral root had been cut. *C*, field potentials evoked by nerve stimulation at 0·5 Hz in normal solution. *D*, after removing Ca^{2+} from the perfusing medium. Stimulus frequencies are 0·5 Hz (upper trace) and 10 Hz (lower trace). All records were averaged (100 trials in *C* and 200 trials in *D*). The upper trace in *E*, intracellular recording from a L5 motoneurone in a 3-day-old rat. Stimulation was applied to the L5 dorsal root. The summit of the e.p.s.p. is not seen in this record. The lower trace was recorded with the electrode tip just outside the motoneurone. Both records in *E* were averaged (110 trials). Positivity upward in *A–E*. *F*, frequency histogram of the interval between the positive peak of the prepotential and the onset of e.p.s.p.s evoked by dorsal root stimulation in 0–3-day-old rats. Dots indicate stimulus artifacts.

Monosynaptic nature of e.p.s.p.s. To examine the synaptic linkage from the primary afferents to the motoneurones, presynaptic volleys elicited by stimulation of the afferent fibres were recorded extracellularly in the motor nucleus and their latencies were compared with those of trans-synaptically evoked field potentials or of e.p.s.p.s.

Fig. 5*A–B* represents field potentials recorded extracellularly in the spinal cord following stimulation of the medial gastrocnemius nerve. After the electrode tip was guided into the medial gastrocnemius motor nucleus by monitoring the antidromic field potential evoked by medial gastrocnemius nerve stimulation at high frequency (*A*), the ventral root was transected. Orthodromically evoked field potentials in the

motor nucleus are shown in *B*. The early portion of the field potentials persists at high frequency of stimulation (5 Hz), while the late portion is depressed markedly (lower trace, compared with upper trace in *B*).

Fig. 5*C* and *D* show orthodromically evoked field potentials in the lateral gastrocnemius-soleus motor nucleus before (*C*) and after (*D*) synaptic transmission was interrupted by removing Ca^{2+} ions from the perfusing solution. Only a small early potential remained after the substitution of solution and it did not change at higher frequency (*D*). These results indicate that the early component represents the presynaptic volley and the late one the trans-synaptically evoked potential. The latencies of the afferent volleys were 23·5 and 20·0 ms in the two experiments. The trans-synaptic potential started less than 1 ms after the volleys arrived in the motor nucleus.

The presynaptic volleys were more clearly observed when the dorsal root was stimulated. Fig. 5*E* (upper trace) shows potentials, evoked by stimulation of the L4 dorsal root, in an L4 motoneurone identified by ventral root stimulation. An e.p.s.p. follows a small potential, which is also seen extracellularly (lower trace). This potential remained after Ca^{2+} ions were removed from the perfusing solution and presumably, therefore, represents the presynaptic volley. The interval between the positive peak of the prepotential and the onset of the e.p.s.p. was measured in fourteen motoneurones. The values ranged between 0·3 and 0·9 ms ($0{\cdot}56 \pm 0{\cdot}16$ ms) as summarized in Fig. 5*F*. Considering the low temperature of the isolated preparation (25 ± 1 °C), it is concluded that the e.p.s.p.s are evoked monosynaptically.

The onset and development of spinal reflexes in the fetus

The results described above indicate that the monosynaptic stretch reflex loops are formed before birth. Thus we further investigated the reflex pathway in the fetus. As it was very difficult to obtain stable intracellular recordings from triceps surae motoneurones in the fetal spinal cord, the reflex response evoked by muscle stretch or electrical stimulation of nerves was examined using e.m.g. recordings from the triceps surae muscle in fetuses of various embryonic days. We also studied the cutaneous reflex response in flexor muscles in these preparations.

Afferent discharge

Afferent discharges evoked by stretch of the triceps surae muscle were examined in the sciatic nerve in thirteen fetuses; two at embryonic day 17·5, four at embryonic day 18·5, three at embryonic day 19·5, two at embryonic day 20·5 and two at embryonic day 21·5. Sample records are shown in Fig. 6 (*A* at embryonic day 20·5; *B* and *C*, at embryonic day 18·5). Ramp-and-hold stretch elicits clear phasic discharges, which augment as the amplitudes of stretch increases; see Fig. 6*A* and *B*. A trace of tonic discharge is seen during stretches of 150 μm. Almost all components of the recorded potentials in *A* and *B* represent nerve discharges and not mechanical artifacts, because they disappeared after conduction in the tibial nerve was blocked by topical application of local anaesthetic (*C*).

Phasic afferent discharges as exemplified in Fig. 6 were observed in all preparations examined. The thresholds of stretch for evoking the discharges ranged on and after embryonic day 18·5 between 30 and 100 μm; there was no systematic difference in

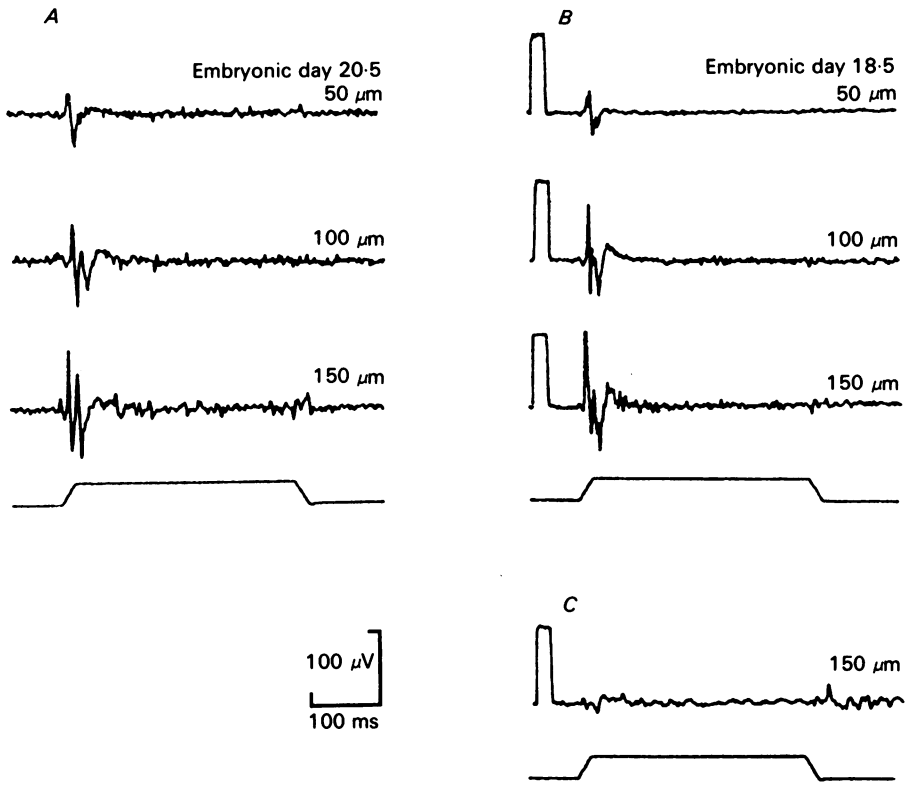

Fig. 6. Afferent discharges evoked by ramp-and-hold stretch of the triceps surae muscle in the fetus. *A*, at embryonic day 20·5. *B* and *C*, at embryonic day 18·5. The discharges were recorded from the sciatic nerve. Positivity upward. Amplitudes of stretch are 50, 100 and 150 μm in the top, middle and bottom trace, respectively, in *A* and *B*. *C*, after application of xylocaine to the tibial nerve at the popliteal fossa. The amplitude of stretch is 150 μm. The lowermost trace in *A–C* shows the displacement.

the thresholds at different ages. At embryonic day 17·5, the youngest stage in this study, phasic nerve discharges were observed in the sciatic nerve following muscle stretches of more than about 100 μm. At this stage, however, we could not separate the triceps surae muscle from other shank muscles. The initial length of the triceps surae muscle could not be set comparable to that at later stages.

Reflex responses in the triceps surae muscle

Reflex effects of brief or ramp-and-hold stretch of the triceps surae muscle were examined using e.m.g. recordings from the muscle in twenty-five fetuses at embryonic day 18·5–20·5. The results are summarized in Table 1. At embryonic day 18·5, muscle stretch did not evoke any reflex responses in the stretched muscle even when the amplitude of stretch was increased to 200 μm as shown in Fig. 7*D*. In half of the preparations at embryonic day 19·5, a reflex e.m.g. response was observed following brief stretches of less than 100 μm, as shown in Fig. 7*A*. Such phasic responses were found in all five preparations examined at embryonic day 20·5. No tonic responses

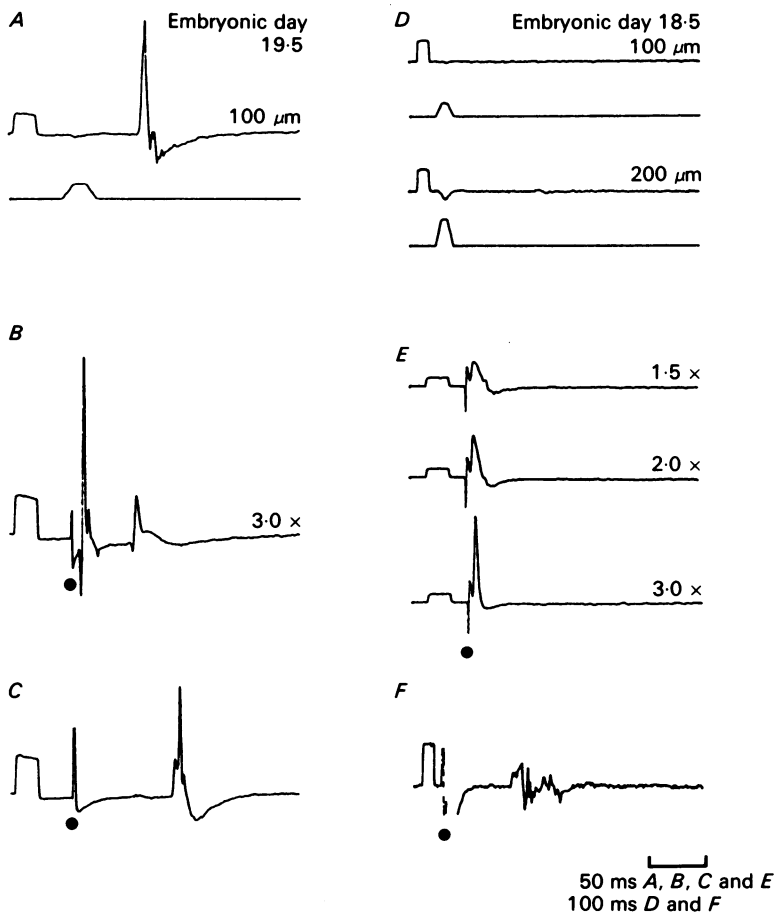

Fig. 7. Spinal reflexes in the fetus. *A–C*, a preparation at embryonic day 19·5. *A* (upper trace) and *B*, e.m.g. responses in the triceps surae muscle evoked by brief stretch (100 μm) of the muscle and by stimulation of the sciatic nerve (3·0 times nerve threshold), respectively. *C*, e.m.g. responses in the biceps femoris muscle evoked by stimulation (1 ms, 10 V) of the plantar skin. *D–F*, a preparation at embryonic day 18·5. *D*, e.m.g. of triceps surae muscle following muscle stretch of 100 μm (upper) and 200 μm (lower). *E*, e.m.g. of the triceps surae muscle following sciatic nerve stimulation. Stimulus intensities are 1·5 (upper), 2·0 (middle) and 3·0 (lower) times nerve threshold. *F*, e.m.g. of the biceps femoris muscle following plantar skin stimulation. Calibration pulse: 100 μV in *A–C*, 50 μV in *D–F*. Dots in *B*, *C*, *E* and *F* indicate stimulus artifacts.

were observed during maintained stretch. Mean latencies of the phasic responses were 74·9 ms at embryonic day 19·5 and 74·4 ms at embryonic day 20·5. These values are similar to those observed in the new-born rat (Table 1).

In two rats at embryonic day 20·5, the threshold of stretch for evoking the reflex response was compared to that for evoking afferent discharges in the same preparations. The values for the former were 40 and 50 μm, while those for the latter were 30 and 40 μm, respectively. It is therefore suggested that impulses in afferent fibres with low mechanical threshold evoke the stretch reflex in the fetus as in the new-born rat.

Effects of electrical stimulation of the sciatic nerve on the triceps surae muscle were also studied in fetuses of different ages. At embryonic day 18·5, only direct muscle responses were evoked even if stimulus intensities were changed systematically as shown in Fig. 7*E*. At embryonic day 19·5, however, stimulation of the sciatic nerve produced a reflex response (Fig. 7*B*) in about half of preparations. Similar reflex e.m.g. responses were observed in all preparations at embryonic day 20·5 (Table 1).

TABLE. 1. Frequency of occurrence (left column) and latencies (right column) of spinal reflexes following three types of stimulation at various ages

	Muscle stretch (t.s.)		Stimulation of the sciatic nerve (t.s.)		Stimulation of the plantar skin (b.f.)	
Ages	%	Mean ± S.D.(ms)	%	Mean ± S.D.(ms)	%	Mean ± S.D.(ms)
E18·5	0	—	0	—	100	121·7 ± 34·3
	(n = 5)		(n = 6)		(n = 6)	(n = 6)
E19·5	50	74·9 ± 7·2	44	54·6 ± 7·1	100	87·5 ± 20·4
	(n = 10)	(n = 5)	(n = 9)	(n = 4)	(n = 16)*	(n = 9)
E20·5	100	74·4 ± 5·0	100	57·0 ± 4·1	100	101·5 ± 34·0
	(n = 3)	(n = 3)	(n = 4)	(n = 4)	(n = 7)*	(n = 3)
New-born (0–1-day-old)	100	69·7 ± 9·2	100	56·9 ± 7·5	100	133·2 ± 14·3
	(n = 7)	(n = 7)	(n = 5)	(n = 5)	(n = 7)*	(n = 3)

Triceps surae (t.s.), biceps femoris (b.f.); recorded muscle.
E, embryonic day.
n, number of preparations examined.
* The result obtained by behavioral observation is also included.

The effect of muscle stretch and electrical stimulation of the sciatic nerve was compared in the same preparations (fifteen rats). In eight fetuses (two at embryonic day 19·5, five at embryonic day 20·5 and one at embryonic day 21·5), both natural and electrical stimulation evoked a reflex response in the triceps surae muscle (Fig. 7*A* and *B*). In the other seven fetuses (five at embryonic day 18·5 and two at embryonic day 19·5), neither stimulation evoked any reflex responses as shown in Fig. 7*D* and *E*. Mean latencies of the reflex response evoked by stimulation of the sciatic nerve were 54·6 ms at embryonic day 19·5 and 57·0 ms at embryonic day 20·5. These values are essentially the same as those found in the new-born rat (Table 1).

Cutaneous reflex responses in flexor muscles

Pinching or electrically stimulating the plantar skin produced reflex activities in flexor muscles in all the fetuses (embryonic days 18·5–20·5) and new-born rats (0–1-day-old) examined (Table 1). Fig. 7*C* and *F* demonstrate the reflex responses in knee flexor muscles evoked by stimulation of the plantar skin with a pulse of short duration (1 ms) at embryonic day 19·5 and at embryonic day 18·5, respectively. Similar muscle responses were also observed in ankle flexor muscles in all the cases examined. Furthermore, we observed behaviourally the effect of pinching the plantar skin in earlier stage fetuses. A withdrawal movement of the ipsilateral hind limb, indicating the operation of a flexion reflex, was evoked in four out of eleven isolated preparations at embryonic day 17·5, but not at all at embryonic day 16·5 (n = 7).

Latencies of the reflex e.m.g. responses in flexor muscles were very variable. The

values changed by up to about 10 ms from trial to trial in individual preparations. The mean values were, however, always much larger than those for the reflex responses evoked by either muscle stretch or sciatic nerve stimulation in fetuses of any age as well as in the new-born rat, as summarized in Table 1.

DISCUSSION

The present study has demonstrated that reflex e.m.g. responses in ankle extensor muscles can be evoked by muscle stretch as early as embryonic day 19·5. Intracellular recordings from motoneurones in new-born rats have shown that afferent volleys in the muscle nerve produce monosynaptic e.p.s.p.s, which are large enough to generate an action potential. It is thus reasonable to conclude that the reflex e.m.g. response evoked by muscle stretch is transmitted via the monosynaptic reflex pathway in the new-born rat. Although we have not performed intracellular recordings from motoneurones in the prenatal preparation, it is strongly suggested that the reflex e.m.g. response evoked by muscle stretch in the fetus is also transmitted via the same reflex pathway for the following reasons. In both new-born and fetal rats, reflex responses in the stretched muscle and afferent volleys in the muscle nerve were evoked with the same low mechanical threshold. Furthermore, the latencies of the reflex responses evoked in the triceps surae muscle by electrical nerve stimulation were similar and were definitely shorter than those of the polysynaptic cutaneous reflex in knee flexor muscles in both groups of animals (Table 1).

Formation of the reflex pathway

The afferent discharge following muscle stretch was observed even at embryonic day 18·5 and its mechanical threshold was as low as that for evoking the reflex response at later stages. Electron microscopic investigations have shown that detectable muscle spindles first appear at embryonic day 19·5 in the lower hind limb muscle of the rat (Milburn, 1973). However, non-myelinated sensory nerve terminals make contact with undifferentiated single myotubes in gastrocnemius muscle and the terminals possess many of the ultrastructural features of the annulospiral endings of mature spindles even at embryonic day 18·5 (Landon, 1972). It is likely that the sensory nerve terminals have already differentiated so as to be responsive to small muscle stretches at this stage.

Muscle stretch did not evoke the reflex e.m.g. response at embryonic day 18·5, though it elicited the afferent discharge. There is a possibility that the number of muscle afferents activated by muscle stretch is small and the peripheral input is too feeble to activate motoneurones. However, even electrical nerve stimulation failed to evoke the reflex response at embryonic day 18·5. This was also the case in some preparations at embryonic day 19·5 in which the reflex response was not evoked by muscle stretch. According to a recent morphological study by means of horseradish peroxidase staining of sensory fibres, connexions between primary afferents and motoneurones in the thoracic spinal cord begin to form at about embryonic day 18·5 in the rat (Smith, 1983). Similarly, in the rat lower lumbar cord (L4), a large fraction of the afferent fibres begin to project to the motor nucleus at embryonic day 18·5, although a few afferent fibres reach the nucleus even at embryonic day 17·5 (N. Kudo

& T. Yamada, unpublished observation). We therefore suggest that the lack of the stretch reflex response at embryonic day 18·5 is mainly due to immature – if any – synaptic transmission from muscle afferents to motoneurone and that the synaptic effect becomes large enough to activate motoneurones at embryonic day 19·5.

Saito (1979) has investigated electrophysiologically the onset and development of the segmental reflex using the isolated lumbar cord preparation of the rat fetus. He has suggested that the monosynaptic reflex discharge in the lumbar ventral root evoked by dorsal root stimulation appears first at embryonic day 17·5, based upon the similarity of the wave form of the reflex response to that recorded in older fetuses and new-born rats. As in his experiments the segmental reflex was examined at the L3 level, one or two segments rostral to the spinal level of the triceps surae motor nucleus (Nicolopoulos-Stournaras & Iles, 1983); and because the reflex response evoked by dorsal root stimulation was recorded in the ventral root, his results cannot be directly compared with those in the present study. The earlier onset of the monosynaptic reflex response may indicate that maturation of synaptic transmission from muscle afferents to the motoneurones innervating proximal muscles occurs earlier than to those innervating distal muscles, e.g. the triceps surae muscle. However, the possibility cannot be excluded that the reflex discharge at embryonic day 17·5 is evoked polysynaptically. In his experiments, the segmental latency and the time to peak of the early components of reflex responses changed significantly between embryonic days 17·5 and 19·5 (see Table 1 in Saito, 1979).

The present study has also revealed reflex e.m.g. responses in flexor muscles, evoked by natural and electrical stimulation of the plantar skin in the isolated preparation. It is strongly suggested that the reflex responses in flexor muscles are evoked polysynaptically because the latencies are much longer than those for the stretch reflex response and the values are variable from trial to trial in individual preparations. Such cutaneous reflexes were first apparent at embryonic day 17·5 in the present study. This is consistent with the result obtained from *in situ* experiments in the rat fetus (Narayanan *et al.* 1971). In their behavioural observations, a motor response was evoked by tactile stimulation to the skin of the hind limb region at embryonic day 17 (comparable to embryonic day 17·5 in the present study) and in older fetuses, but not at embryonic day 16. That the polysynaptic reflex pathway forms earlier than the monosynaptic one in the rat spinal cord has been also suggested by Saito (1979). In his study, however, stimulation of the dorsal root was first effective in evoking reflex discharges with long segmental latencies in the ventral root as early as embryonic day 15·5. In the present experiments no reflex responses to pinching the plantar skin appeared in hind limb muscles at embryonic day 16·5. The production of responses as early as day 15·5 may require large afferent volleys which might be more easily produced by dorsal root stimulation than by local stimulation of the skin.

Properties of the stretch reflex pathway during the development

A characteristic property of the stretch reflex response in the triceps surae in the immature rat is the lack of a tonic component during maintained stretch. Only phasic reflex responses were evoked by ramp-and-hold stretches of large amplitude in new-born and fetal rats. The lack of tonic responses seems to be due mainly to the properties of the afferent discharges: ramp-and-hold stretch elicited only phasic

discharges in most afferent fibres. One may argue that this might be attributed to the metabolic state of the isolated preparations used in the present study, because the tonic component of the afferent discharges evoked by stretch is severely affected by restriction of the blood supply to the muscle in *in situ* experiments (Matthews, 1933). This is, however, unlikely because many afferent fibres are tonically active during maintained stretch of the triceps surae muscle in isolated preparations from 7–10-day-old rats (N. Kudo, unpublished observations). Furthermore, Skoglund (1960*c*) has shown that tonic afferent discharges are not evoked during maintained stretch of the gastrocnemius muscle in the anaesthetized new-born kitten, in which circulation has been kept normal. It is possible, therefore, that the afferent discharge of the muscle receptor in response to muscle stretch is phasic in character in the immature mammal. In the new-born rat, even if tonic discharge was elicited in the sensory nerve innervating the muscle spindles, it could not evoke a sustained increase in the excitability of the target motoneurones because the amplitude of monosynaptic e.p.s.p.s was very susceptible to the repetition rate. Only a trace of e.p.s.p. was observed at a stimulus frequency of 10 Hz (Fig. 4*D*), while the total duration of the monosynaptic e.p.s.p. was less than 50 ms (Fig. 4).

It is known that the monosynaptic e.p.s.p. evoked by muscle afferents in motoneurones persists at high rates of repetition in the adult cat (Curtis & Eccles, 1960). The amplitude of the e.p.s.p. is only depressed to 70–85 % of the control size as the stimulus frequency is raised to 4–20 Hz. The persistence of the monosynaptic e.p.s.p. during high frequencies of stimulation seems to change drastically during the development of the reflex pathway, because the amplitude of the e.p.s.p. is depressed to about 40 % of control size at a stimulus frequency of 10 Hz in a 32-day-old kitten (Eccles & Willis, 1965) and to less than 10 % at 10 Hz in younger, new-born rats (Fig. 4*D*). The present study shows that the size as well as the shape of the field potential representing presynaptic impulses in the motor nucleus remains nearly constant at stimulus frequencies as high as 10 Hz. Thus the prominent depression of the e.p.s.p. at 10 Hz would not be due to the long refractory period of the immature sensory nerve (Hursh, 1939), but might result from properties of the immature presynaptic terminal, such as depletion of the available store of transmitter, a failure of invasion of sensory impulses into every terminal or a decrease in the probability of transmitter release at high repetition rates.

The specific spatial pattern of connexions between muscle afferents and motoneurones innervating various muscles of hind limb has been shown in the cat (Eccles, Eccles & Lundberg, 1957; Eccles, Eccles, Shealy & Willis, 1962; Burke, 1968; Hongo, Lundberg, Phillips & Thompson, 1984) and in the baboon (Hongo *et al.* 1984). Generally, the largest monosynaptic e.p.s.p. is evoked by impulses in the homonymous muscle afferent fibres. A similar dominance of homonymous muscle afferents has also been observed in motoneurones of 19–45-day-old kittens (Eccles *et al.* 1963) and now in new-born rats (present study). Frank & Westerfield (1983) have shown that the pattern of innervation is specific from the beginning of the formation of the monosynaptic reflex pathway in the brachial spinal cord of the frog. It has been also suggested that the specific pattern of innervation is quite rigidly determined at an early developmental stage in the embryonic chick spinal cord, because the pattern of the connexion is not compensated after a lesion in the neural crest (Eide, Jansen &

Ribchester, 1982). It is uncertain whether this is also the case for the development of the monosynaptic reflex pathway in the mammal. However, the present study makes it clear that the specific pattern of innervation of the triceps surae motoneurones by the muscle afferents has been established by birth in the rat. Even if inappropriate connexions occur at the beginning of synapse formation, they will be corrected during the last two or three days of gestation in the rat.

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