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Muscle fibre types and innervation of the chick embryo limb following cervical spinal cord removal

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SUMMARY

Several segments of spinal cord were removed from the cervical regions of stage-13 or -14 (day-2) chick embryos. After further incubation to day 17 or 18, the patterns of end-plate distribution and ATPase typing of muscle fibres in the anterior and posterior *latissimus dorsi* and the *ulnimetacarpalis dorsalis*, and the ATPase typing of the forearm muscles were examined. No differences from control embryos were found. The embryos had normal numbers of lateral motor column motoneurons in both the brachial and lumbar enlargements and the positions of motoneurons supplying the biceps as identified with retrograde horseradish peroxidase labelling were consistent with the normal patterns of motor projection into the limb. These results show that the fibre typing of limb muscles and their patterns of innervation are independent of descending inputs until just before hatching in the chick.

INTRODUCTION

Transection of the spinal cord of neonatal and adult mammals greatly affects the properties of skeletal muscle fibres supplied by spinal motoneurons caudal to the lesion. In neonates, slow muscles do not develop their normal properties, and in adults slow muscles develop fast muscle properties (Buller, Eccles & Eccles, 1960; Karpati & Engel, 1968; Grimby, Broberg, Krotkiewska & Krotkiewski, 1976; Gallego, Huizar, Kudo & Kuno, 1978; Rubinstein & Kelly, 1978). Fast muscle fibres are relatively unaffected. These effects are probably mediated by the alteration of function caudal to the lesion, which may give rise to increased or decreased peripheral motor activity, depending on species and maturity (Barcroft & Barron, 1937; Wang & Lu, 1940; Barron, 1941; Buller *et al.* 1960; Sims, 1962; Stelzner, 1975; Forssberg, Grillner & Halbertsma, 1980; Forssberg, Grillner, Halbertsma & Rossignol, 1980; Forehand & Farel, 1982; Smith, Smith, Zernicke & Hoy, 1982). In the chick embryo, alteration of behaviour caudal to spinal transection can first be demonstrated at approximately the tenth day of incubation (E10) (see Oppenheim 1975 for review) and alteration of the electrical activity of the cord caudal to the lesion can be seen at least as early as E13 (Provine & Rogers, 1977). Transection increases the duration and decreases the frequency of the cyclic bursts of activity seen in embryos showing that supraspinal inputs only modulate the spinally generated activity at these stages (Oppenheim, 1975;

Key words: chick embryo, spinal cord transection, muscle fibre type, motoneurons.

Provine & Rogers, 1977). Alteration of activity after E10 induced by exogenous electrical stimulation at 0·5 Hz changes the muscle fibre typing of the peripheral musculature towards slow properties within four days (the focally innervated posterior *latissimus dorsi* muscle becomes multiply innervated to some extent and contains many more acid-stable fibres than normal) (Renaud, Le Douarin & Khaskiye, 1978; Toutant, Toutant, Renaud & Le Douarin, 1979; Toutant *et al.* 1980*b*; Toutant *et al.* 1981; Renaud, Gardahaut, Rouaud & Le Douarin, 1983). Thus, it is possible that transection of the spinal cord in embryos may alter the development of muscle fibre types in a way similar to a) the change seen in postnatal mammals, b) to the change seen following exogenous electrical stimulation, or c) there may be no effect at all depending on the influence of transection on whatever aspect of spinal cord activity is controlling muscle fibre type.

Further questions arise in relation to motoneuron numbers and the formation of motor pools. Pharmacological blockade of motor function during development of the embryo causes an increased number of motoneurons to survive the period of normal motoneuron death (Pittman & Oppenheim, 1978, 1979; Laing & Prestige, 1978; Creazzo & Sohal, 1979; Harris & McCaig, 1984). It is therefore conceivable that descending inputs onto the spinal motoneurons may alter their survival through control of spinal function. In addition, through influencing the patterns of motoneuron survival, the final patterns of motor projection into the limb could be affected. It has been hypothesized that motoneurons mismatched for fast/slow properties die during normal motoneuron death (Laing & Lamb, 1983*b*; Lamb, 1984; Gauthier, Ono & Hobbs, 1984). Any change in muscle fibre distribution could therefore be accompanied by alteration of patterns of motoneuron survival. These could be reflected in the numbers of motoneurons surviving and the projections of individual motoneuron pools.

To investigate these questions we have examined various parameters of peripheral motor development following removal of several segments of cervical spinal cord.

METHODS

Fertilized White Leghorn eggs were incubated in a humidified forced-draught incubator at approximately 39°C. On the second day of incubation (E2), the eggs were windowed. The embryos were staged according to Hamburger & Hamilton (1951) and operations were performed on embryos of stages 13 and 14 which is prior to limb bud innervation (Roncali, 1970). Evans Blue dissolved in Hanks Balanced Salt Solution containing 50 i.u. ml^{-1} penicillin and 50 $\mu g\,ml^{-1}$ streptomycin buffered with HEPES/NaOH and sterilized by passage through a millipore filter (0·2 μm) (all Flow laboratories) was injected into the yolk sac below the embryo to improve visualization of the embryonic structures. The embryonic membranes were torn open. Using electrolytically sharpened tungsten needles, the neural tube was cut transversely at the level of approximately the 13th somite which is destined to become the ninth spinal segment (Hamburger, 1946). The brachial plexus extends from the 12th to the 17th spinal segments in early embryonic stages, and from the 13th to the 17th segments in late stages (Roncali, 1970; Pettigrew, Lindeman & Bennett, 1979) and thus should not be affected by the operation. Longitudinal cuts were made rostral to the transection between the neural tube and the somites

and the piece of neural tube thus freed was then sucked out with a micropipette. Four to six segments were removed in order to reduce the probability of axons subsequently growing across the gap. Care was taken to ensure that no small pieces of neural tube were left in the operated region since this was found in preliminary experiments to increase the proportion of unsuccessful operations. The window was sealed with adhesive cellophane tape and the egg returned to the incubator until E17 or E18.

At E17, the right biceps muscle of some of the embryos was injected with horseradish peroxidase (HRP) (Type VI Sigma) as described previously (Laing & Lamb, 1983*a*). At E18, embryos were killed by decapitation at the level of the rostral mid-brain. The brachial enlargement of HRP-injected embryos was fixed in 2·5% glutaraldehyde and processed to reveal labelled motoneurons (Laing & Lamb, 1983*a*). The lumbar enlargement of these embryos and both the brachial and lumbar enlargements of embryos not injected with HRP were fixed in Carnoy's solution, dehydrated in alcohol, cleared in cedarwood oil, embedded in paraffin wax, sectioned at 12 μm and stained with haematoxylin and eosin for motoneuron counts. Motoneurons of the lateral motor column (LMC) were counted in every tenth section. Only cells containing one or more nuclei were counted with no correction for double counting.

The anterior and posterior *latissimus dorsi* muscles (ALD and PLD) and the wrist region (containing the *ulnimetacarpalis dorsalis* (UMD)) of one wing were stained for acetylcholinesterase activity by the method of Karnovsky & Roots (1964). These muscles and the forearm of the other wing were frozen in OCT freezing compound using isopentane (FLUKA) cooled by liquid nitrogen, sectioned at 16 μm in a cryostat and stained for ATPase activity (Dubowitz & Brooke, 1973). The ALD and PLD were frozen stretched out on pieces of lens tissue to ensure good transverse sections.

RESULTS

Operations were performed on 59 embryos. Of these, 33 died prior to E17/E18, 3 of the 26 surviving embryos had incomplete gaps and one had a gap too close to segment 13. In embryos with successful operations, mechanical stimulation of the head elicited no response in the torso *in ovo*, and on dissection the gap was seen to be complete (Fig. 1). In a number of the embryos the vertebral column as well as the spinal cord was discontinuous at the gap (see also Hamburger, 1946).

Acetylcholinesterase and ATPase staining

Acetylcholinesterase staining of the ALD, PLD and UMD of operated embryos was grossly similar to that of normal embryos (Fig. 2). The ALD and the slow head of the UMD showed a distributed pattern with many small sites of cholinesterase activity along the entire length of the muscle, whereas the PLD and the fast head of the UMD had fewer bands of larger end plates.

The ALD of both operated and normal embryos consisted almost entirely of acid and alkali stable fibres with only a very small percentage of acid labile/alkali stable fibres (Fig. 3A–D) mainly along the anterior edge of the muscle as in the normal chick (Toutant, Toutant, Renaud & Le Douarin, 1980*a*).

The PLD, in both control and operated embryos, contained mainly acid-labile fibres with only a small and variable percentage of acid-stable fibres (Fig. 3E–H).

In the UMD the 'slow' head contained predominantly acid- and alkali-stable fibres with a variable percentage of acid-labile fibres and the 'fast' head was

predominantly acid labile with a small and variable percentage of acid-stable fibres near the 'slow' head (Fig. 4).

In the forearm, the patterns were similar to normal. Acid-stable fibres predominated in the *brachialis* muscle while the other muscles contained their usual proportions and distributions of acid-stable and acid-labile fibres.

HRP tracing of biceps motor pool

The biceps was injected with HRP in eight embryos with successful operations. Three of these embryos died before the next day but good motoneuron pools were mapped in the other five. The positions of the motor pools were normal in all cases in both the rostrocaudal (Fig. 5) and mediolateral (Fig. 6) axes.

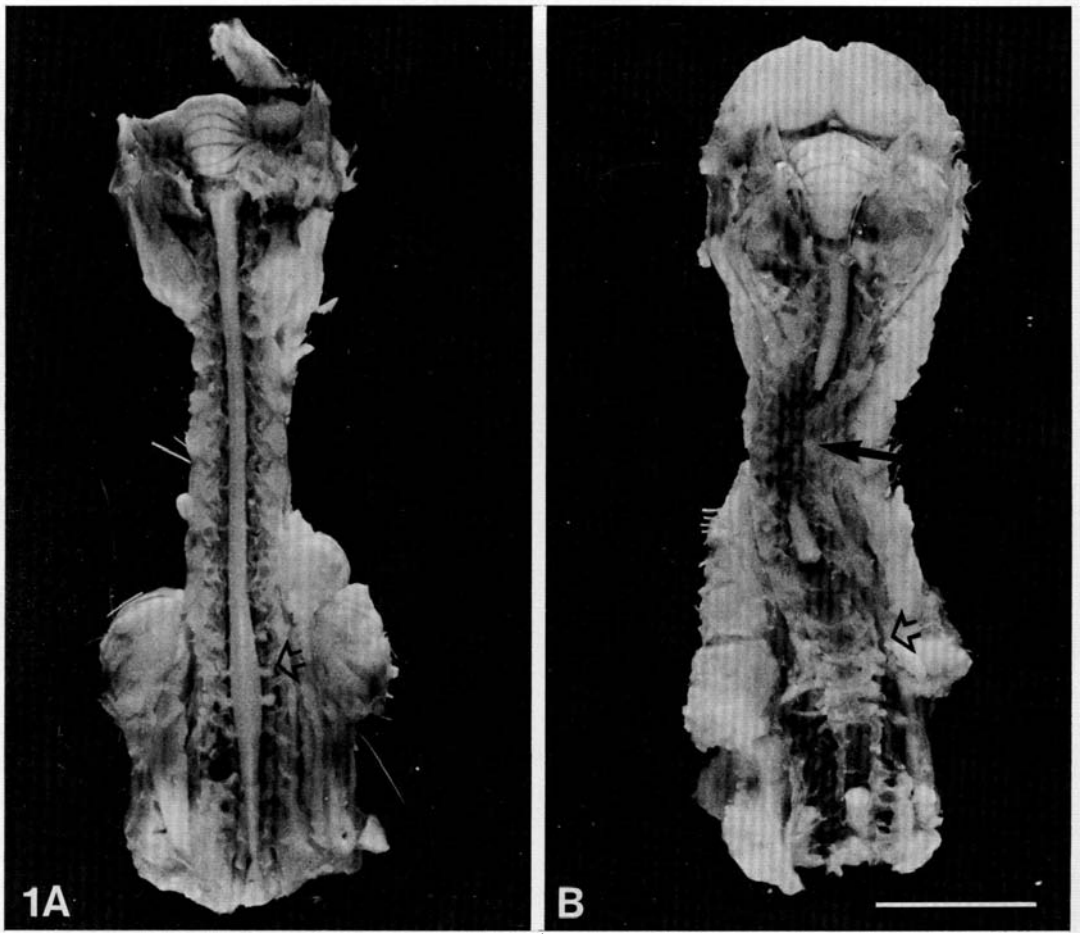

Fig. 1. Dorsal view of spinal cord from normal (A) and operated (B) embryos at E18 following removal of cord segments at E2. Open headed arrow in (A) points to brachial enlargement which has been removed in (B) for motoneuron counts. Arrow in (B) points to lesion ('gap') between brain and spinal cord. In many of the embryos the damage extended from just caudal to the hind brain through to segmental nerve nine or ten. Bar = 1 cm.

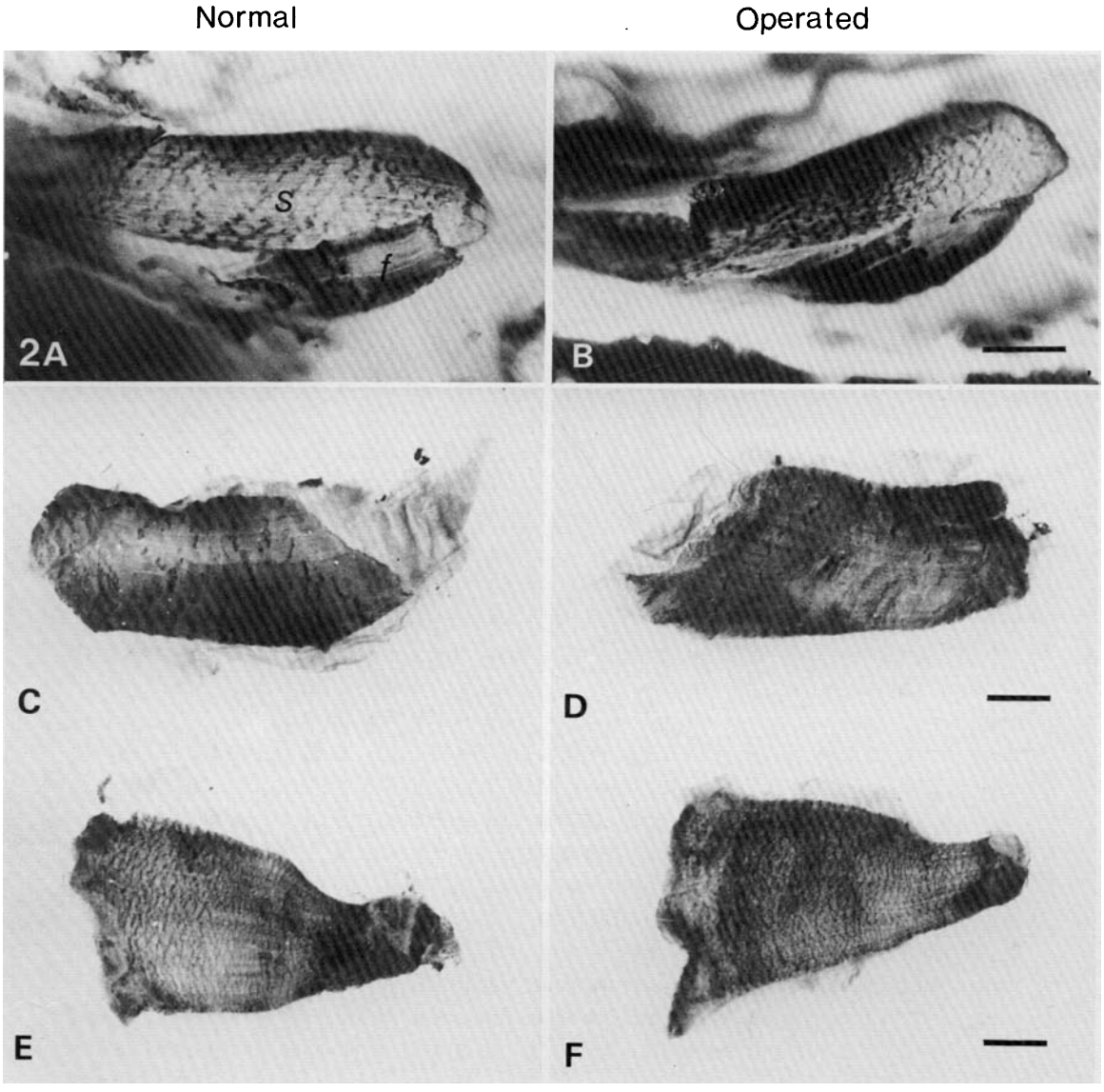

Fig. 2. Acetylcholinesterase staining of whole muscles from wings of operated and control embryos at E18. (A,B) *ulnimetacarpalis dorsalis*, (C,D) posterior *latissimus dorsi*, (E,F) anterior *latissimus dorsi*. The slow head (*s*) of the UMD and the ALD show the typical distributed end-plate pattern of slow muscles while the fast head (*f*) of the UMD and the PLD show the focal distribution of fast muscles. Little or no difference is visible between operated and control embryos. The operated PLD in (C) demonstrates a common problem with the cholinesterase staining of the *latissimus dorsi* muscles in that the staining is patchy where the overlying connective tissue has prevented even penetration. Bar in B = 0·5 mm; bars in D & F = 1 mm.

Table 1.

		Control	Spinalized
Number of lumbar LMC motoneurons		13 096 ± 1183* (n = 16)	12 640 ± 1293 (n = 11)
	Range	11 000 to 15 155	9945 to 14 025
Number of brachial LMC motoneurons		8343 ± 871 (n = 16)	7884 ± 813 (n = 10)
	Range	7330 to 9890	6340 to 8990

* Mean ± standard deviation.

Motoneuron counts

The number of motoneurons innervating the limbs of operated and control embryos at E18 were not significantly different ($P > 0{\cdot}1$ for two-tailed Students t-test and Mann-Whitney U-test) (Table 1). The embryos with the smallest number of motoneurons were embryos which had been classified as 'runts' (considerably smaller than normal) during the gross dissection.

DISCUSSION

Muscle fibre types can be differentiated in chick embryo muscles destined to become almost pure 'fast' or pure 'slow' muscles as soon as they can be recognized as separate entities. In proximal muscles such as the ALD and PLD, the fast and slow fibres can be distinguished at stages 29–30 (E6 to E6.5) (Butler & Cosmos, 1981), and in distal muscles such as UMD, by stages 33–34 (approximately E8) (Laing & Lamb, 1983*a*). The checkerboard pattern of mixed muscles cannot develop fully until the secondary myofibres have matured (from stage 38 (E12) onwards in the chick hind limb (McLennan, 1983)). Even after hatching the ATPase typing of muscles continues to change (Toutant *et al.* 1980*a*). In the present study, the removal of several segments of the cervical spinal cord prior to limb innervation had no significant effect on muscle fibre ATPase typing or end-plate distribution patterns by late embryonic stages. Neither was there any effect on motoneuron survival or on the location of the motor pool to biceps, indicating no major alterations to the normal motor projection patterns. These results demonstrate that supraspinal inputs play little or no part in the control of the early to late embryonic stages of skeletal muscle development and its innervation.

At present there is little published data on the question of the role of supraspinal inputs in controlling embryonic muscle fibre type though G. J. Hausman and colleagues (personal communication) have recently found the ATPase typing of the *semitendinosus* muscle of the foetal pig to be normal on the 110th day of gestation following high cervical transection of the spinal cord on the 45th day of gestation. This complements previous work in which muscle dry weight and nuclear to myofibre ratio were found to be unaffected (Campion, Richardson,

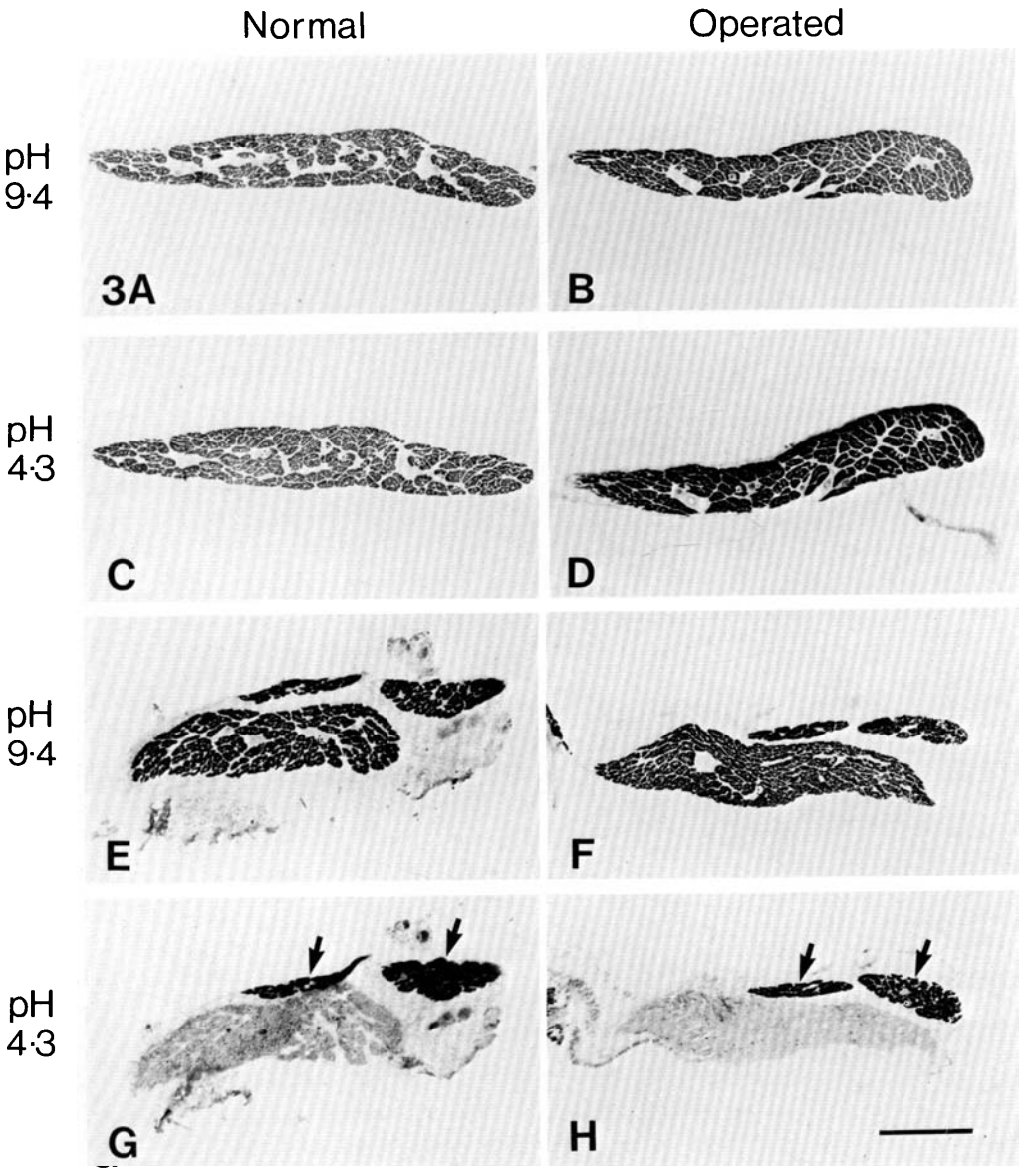

Fig. 3. ATPase staining of transverse sections of control and operated ALD (A–D) and PLD (E–H) following alkaline (pH 9·4) (A,B,E,F) and acid (pH 4·3) (C,D,G,H) preincubation. The ALD of both control and operated embryos consists almost entirely of muscle fibres which are both acid and alkali stable giving dark reaction product at both high and low pH. The PLD is almost exclusively acid labile in both control and operated embryos. The two acid stable muscles adjacent to the PLD are the slow *dorsocutaneous* and *metapagialis* muscles (arrows in (G) and (H)). Bar = 0·5 mm.

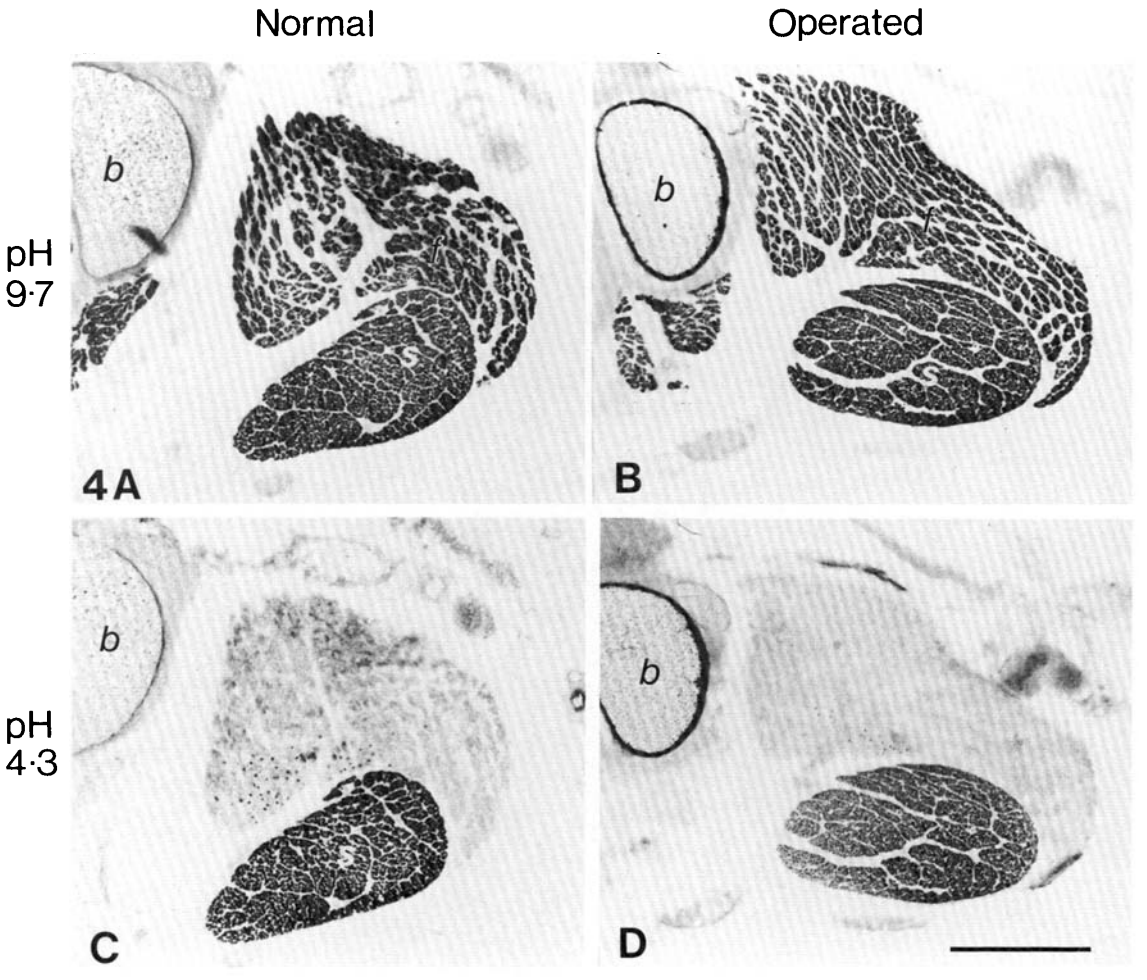

Fig. 4. ATPase staining of transverse sections of UMD in control (A,C) and operated (B,D) embryos. In both cases the fast head (*f*) contains very few acid stable fibres whereas the slow head (*s*) contains mostly acid stable muscle fibres. *b*, bone. Bar = 0·5 mm.

Kraeling & Reagan, 1978). The only changes they found were a reduction in the number and activity of satellite cells and changes in some enzyme levels. These results are in apparent contrast to other reports by Hausman and his colleagues (Kraeling, Rampacek, Campion & Richardson, 1978; Campion, Hausman & Richardson, 1981; Hausman, Campion & Thomas, 1982; MacLarty *et al.* 1984) in which significant changes were observed in some muscles after foetal decapitation. However, these effects were considered to be due to endocrinological changes arising from the loss of pituitary function.

Studies of the developing diaphragm in the sheep foetus lead to similar conclusions to those of the early pig experiments in that transection of the spinal

cord above the level of the phrenic nerve at 114 days of gestation does not alter the weight or histological appearance of the diaphragm at term (Liggins *et al.* 1981). This is in contrast to phrenic nerve section which results in atrophy (Fewell, Lee & Kitterman, 1981). The histochemistry of the diaphragm was not examined in these studies.

However, it is apparent that unlike neonatal and adult mammals (Buller *et al.* 1960; Karpati & Engel, 1968; Grimby *et al.* 1976; Gallego *et al.* 1978; Rubinstein & Kelly, 1978), muscle fibre types are relatively independent of supraspinal inputs during development of at least pig and chick embryos.

Coordinated behaviour dependant on supraspinal control commences at E17 in the chick (Hamburger & Oppenheim, 1967). The consequent changes in muscle usage may be responsible for the further alterations seen in the ATPase staining characteristics of chick muscles which occur around hatching (Toutant *et al.* 1980*a*). Thus, supraspinal inputs may have effects on muscle fibre typing in chicks similar to the effects in postnatal mammals after the period we examined.

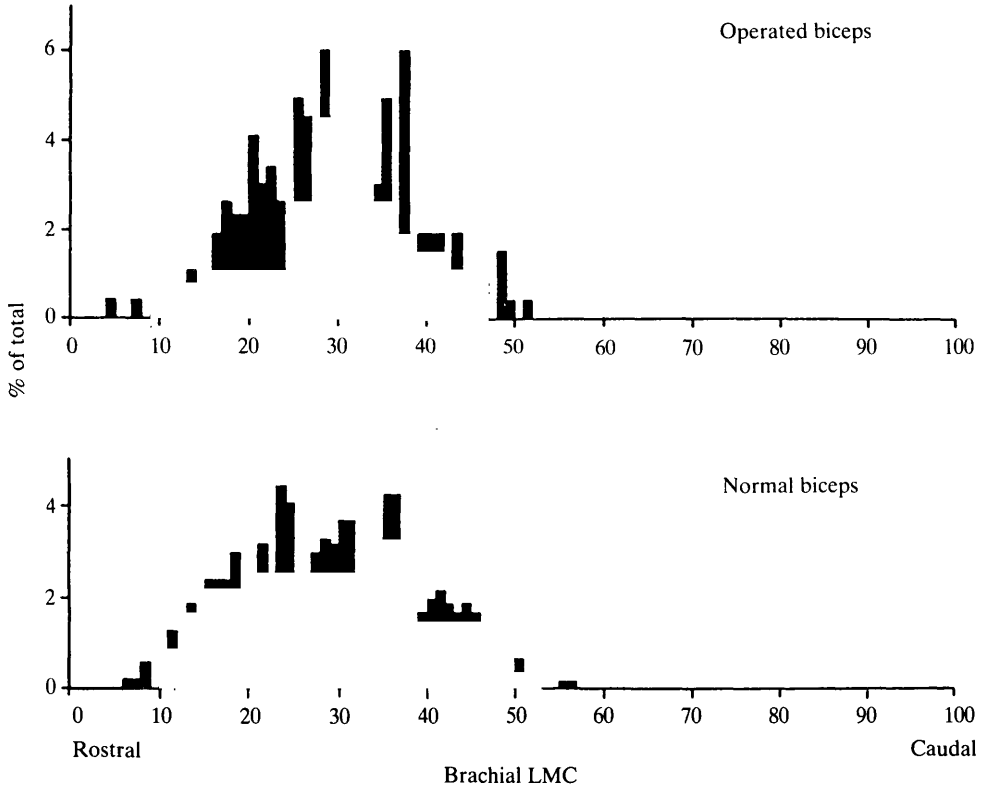

Fig. 5. Rostrocaudal distributions of labelled motoneurons following injection of HRP into the biceps of operated and control chicks. The distributions of the labelled motoneurons are virtually identical. Distributions for individual embryos were normalized before pooling by dividing the LMC into 100 equal parts. Normal: 7 embryos, total of 539 labelled motoneurons; operated: 5 embryos, 266 motoneurons.

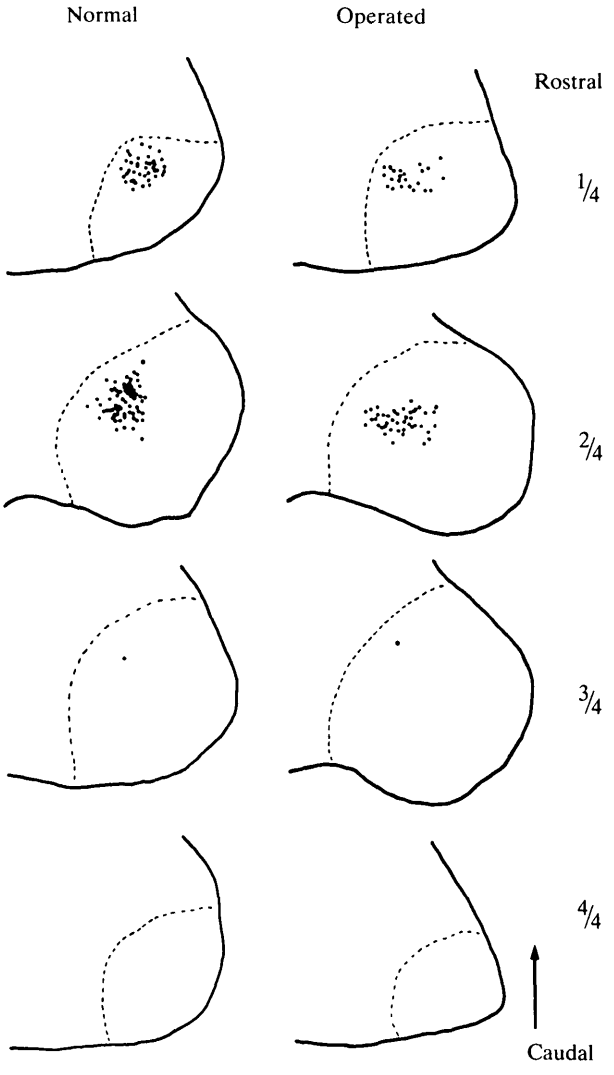

Fig. 6. Mediolateral distributions of HRP-labelled motoneurons in one normal and one operated embryo. The distributions are almost identical. The LMC was divided into four equal parts rostrocaudally and the position of labelled motoneurons within each quarter marked using a camera lucida. Each section was aligned with the drawing using the central canal and the edge of the grey matter as markers.

Our results showing a slight though not significant reduction in LMC motoneuron numbers following cervical spinal cord removal at first sight contrast with a recent report by Okado & Oppenheim (1984) in which they conclude that spinal cord transection reduces motoneuron number late in embryonic development. However, close scrutiny of their results shows that following cervical transection there was no significant reduction in the number of lumbar LMC motoneurons at E16 and a reduction of borderline significance at E18

(0·05 % in a one-tailed test). Taken together the two sets of results show that any reduction in motoneuron number following cervical transection is slight at most at E18. The reductions that Okado & Oppenheim (1984) obtained following thoracic transection, neural crest removal and a combination of these procedures were more marked. This would indicate that propriospinal and dorsal root inputs are of more importance in maintaining LMC numbers than supraspinal inputs, though some of the reduction in motoneuron number following neural crest removal may be due to a lack of muscle spindle targets for beta and gamma motoneurons and not to a reduction in afferent input (Eide, Jansen & Ribchester, 1982).

The mechanisms by which the spinal cord and periphery exercise autonomous control over the initial patterns of motor projections, motoneuron survival, muscle end-plate distributions and muscle fibre types are unknown. But some of these patterns appear to be determined by the limb. This is shown in the normal patterns of early ATPase distributions in aneurogenic limbs (Butler, Cosmos & Brierley, 1982) and in the normal patterns of early and late ATPase and acetylcholinesterase patterns in heterotypically innervated limbs (Laing & Lamb, 1983*b*; Jacob, Christ & Grim, 1983). Muscle fibre types stay normal in the aneurogenic limbs until the muscles atrophy due to lack of innervation (E7 to E16 depending on the muscle (Butler *et al.* 1982)). Changes can however be introduced in all four parameters by interfering with the function of the motor system. Cholinergic blockade prevents motoneuron death (Pittman & Oppenheim, 1978, 1979; Laing & Prestige, 1978; Creazzo & Sohal, 1978; Harris & McCaig, 1984), changes ATPase and myosin typing (Laing, 1982*a*; McLennan, 1983; Gauthier *et al.* 1984), produces distributed innervation in what would normally be focally innervated muscles (Pittman & Oppenheim, 1979; Ding, Jansen, Laing & Tonnesen, 1983) and, in some studies, has been found to have subtle effects on motor projections (the pattern of survival of motoneurons following graded amputations is different in control and paralysed embryos (Laing, 1982*b*)). These observations together with the alteration of muscle fibre type by exogenous stimulation in the embryo (Renaud *et al.* 1978, etc.) suggest that some aspect of the pattern of electrical activity within the spinal cord is one means by which control is exercised over the early development of muscle and its motor innervation.

The electrophysiological studies of Provine & Rogers (1977) and the behavioural studies of Oppenheim (1975) both indicate that the normal cyclic bursts of activity are less frequent and more prolonged following spinal cord transection: the effects being observable as early as E10. The present experiment would indicate that the aspect of activity in the embryonic spinal cord which influences muscle fibre type is insufficiently altered by transection up until E18 to change muscle fibre type.

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