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ACTIVATION PATTERNS OF EMBRYONIC CHICK HIND-LIMB MUSCLES FOLLOWING BLOCKADE OF ACTIVITY AND MOTONEURONE CELL DEATH

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SUMMARY

1. Motoneurone cell death and spontaneous embryonic motility were blocked in chick embryos by daily *in ovo* injections of *d*-tubocurarine from stage 28–36 (E5–10). Isolated spinal cord–hind-limb preparations were prepared from these embryos and movement sequences in response to electrical stimulation of the thoracic cord were assessed, after drug wash-out, by electromyogram (e.m.g.) or muscle-nerve recordings.

2. In embryos in which complete blockade of lumbar motoneurone cell death was later confirmed histologically, flexor and extensor motoneurone pools were found to be activated in alternating bursts as occurs in control embryos. Thus the development of the basic cord circuits responsible for these patterns of motoneurone activation does not require motoneurone cell death. Partial blockade of motoneurone cell death by guanosine 3′,5′-phosphate (cyclic GMP) was also without effect on muscle activation patterns.

3. *In ovo* injection of *d*-tubocurarine or α-bungarotoxin in doses sufficient to block embryonic motility was found to have a direct effect on the spinal cord, preventing the patterned activation of motoneurone pools in alternating bursts. Cords removed from treated embryos behaved similarly to cords in which these drugs were applied acutely in the bath. Minor changes in muscle activation patterns that occurred with chronic drug treatment were also observed in acutely treated cords and appear to be a direct and persistent effect of the drugs on cord circuits.

4. It is possible to conclude that cholinergic circuits within the chick lumbar cord play a role in the normal patterned activation of flexor and extensor motoneurone pools. Systemically applied drugs can have access to these circuits, indicating a need for caution when interpreting the results of drugs applied in this manner to developing embryos.

5. We also conclude that neither the activation of motoneurones in patterned bursts, nor the afferent feed-back from the movements that result, are required to form the basic spinal cord circuits responsible for the activation of extensor and flexor motoneurone pools in alternating bursts.

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INTRODUCTION

Patterned activity occurring in subgroups of developing neurones has been proposed as a means of refining or sharpening neural circuits set up by other mechanisms such as chemo-affinity. In recent years, considerable evidence has been obtained which supports this role for activity, especially in the visual system (Stryker, 1981; Meyer, 1982; Reh & Constantine-Paton, 1985; Boss & Schmidt, 1985). Activity has also been shown to affect the withdrawal of polyneuronal innervation at the neuromuscular junction (Benoit & Changeux, 1975; Brown, Jansen & Van Essen, 1976; O'Brien, Östberg & Vrbová, 1978; Thompson, Kuffler & Jansen, 1979; Brown, Holland & Hopkins, 1981; Thompson, 1983) and recent observations suggest that active terminals may have a competitive advantage over inactive terminals during this process (Ribchester & Taxt, 1984; Ridge & Betz, 1984). In several species, withdrawal of polyneuronal innervation is accompanied by a sharpening of segmental projections within muscles (Brown & Booth, 1983; Bennett & Lavidis, 1984).

Motoneurones innervating the vertebrate limb have for the most part been found to project to appropriate muscles from early on (Landmesser & Morris, 1975; Landmesser, 1978; Lance-Jones & Landmesser, 1981; Hollyday, 1983; Lance-Jones, 1984; Tosney & Landmesser, 1985; Farel & Bemelmans, 1985; but see Lamb, 1976). In the chick, motoneurones have been found to project specifically by stage 30 or E7 (Landmesser & Morris, 1975; Landmesser, 1978; Hollyday, 1983) and even earlier (Lance-Jones & Landmesser, 1981; Tosney & Landmesser, 1985). Thus the distinctive patterns in which embryonic motoneurone pools are activated (Bekoff, 1976), and which develop only at about stage 30–31 (E7–8) (M. O'Donovan & L. Landmesser, unpublished observations and in preparation) would not be expected to play a major role in the matching of motoneurone pools with their appropriate muscles; nor would the process of natural cell death, most of which occurs after stage 30 (E7) (Hamburger, 1975; Chu-Wang & Oppenheim, 1978; see also Oppenheim, 1981).

However, motoneurones must also become functionally integrated into spinal cord circuits by forming a number of specific connexions. Many of these central connexions apparently form during the time that motoneurones are dying in large numbers and during the time that motoneurone pools develop distinctive activation patterns (Bekoff, 1976). It is therefore possible that both processes do play a role in the establishment of specific central connexions.

In the chick embryo it is possible to completely prevent motoneurone cell death by chronic *in ovo* injections of neuromuscular blocking agents such as *d*-tubocurarine (*d*TC) and α-bugarotoxin (α-BTX) (Pittman & Oppenheim, 1979) or guanosine 3′,5′-phosphate (cyclic GMP) (Weil & Greene, 1984; but see Oppenheim & Weil, 1985). To determine if motoneurone cell death, or the patterned activation of muscles that occurs during spontaneous embryonic motility, plays a role in the formation of spinal circuits, we exposed chick embryos to *d*TC or cyclic GMP throughout the cell-death period. We then assessed the ability of spinal cords from these embryos to activate muscles in characteristic patterns by making electromyographic (e.m.g.) or muscle-nerve recordings from isolated spinal cord–hind limb preparations (Landmesser & O'Donovan, 1984*a*) after drug wash-out. A brief report of this work has appeared previously (Landmesser, Szente & Dahm, 1985).

METHODS

White Leghorn chick embryos were incubated until the desired age, staged according to Hamburger & Hamilton (1951) and subjected to the following procedures.

Drug application and assessment of spontaneous motility

For the series in which motility was chronically blocked, a square hole was made in the shell and a transparent window made out of a cover-slip was sealed in place with melted paraffin. In one group of sixty-five embryos, 2–3 mg of *d*TC in 0·1 ml of sterile Tyrode solution was applied daily to the chorioallantoic membrane from stage 28–39 (E6–12). Chronic application of *d*TC results in an increase in mortality, so that only thirty-seven embryos survived to the desired stages. Of these, sixteen were used in the present study and were sacrificed at stage 35½–38. The remainder were allowed to survive to later stages (40–45, E14–21) and were used in a separate study on muscle fibre type differentiation. Five additional embryos received injections of 0·1 ml of sterile Tyrode solution each day and served as controls. In a separate experimental series dibutyryl-cyclic GMP (1 mg/day) was applied in a similar manner to twenty-seven embryos between stages 28–39.

In a few embryos 2–3 mg *d*TC or 75 μg α-BTX per embryo were applied *in ovo* once, 10–15 h before sacrifice. A series of embryos which received no injection or only sterile Tyrode solution was used as controls.

Spontaneous motility has been well characterized in chick embryos by behavioural (Hamburger, Balaban & Oppenheim, 1965; Oppenheim, 1975) as well as electrophysiological (Ripley & Provine, 1972; Bekoff, 1976) observations. As early as stage 30 (E7) embryos begin to move, producing an average of $5 \pm 1·3$ movements/min (mean ± S.D.), which increases to $19 \pm 4·3$ movements/min by stage 36 (E10) (Oppenheim, 1975). Since the intent of the present experiments was not to characterize the blockade of activity in any detail, but simply to confirm that the doses of applied drugs (*d*TC and α-BTX) blocked motility as previously described, a similar paradigm to those used in earlier studies was employed; the number of hind-limb movements during a 3 min observation period was counted each day. In previous studies this has been found adequate to characterize motility, and in the present study a return toward normal values of activity following cessation of drug application was clearly correlated with a loss of the neurones rescued by the blockade. However, in some embryos, motoneurone cell death was only partially blocked, although motility block appeared essentially complete. It is possible that subtle differences in the amount of spontaneous activity occurred between the embryos with complete and those with only partial block of motoneurone cell death, and would have been detected by more extensive periods of observation. For this reason, only the animals in which complete block of motoneurone cell death was achieved were analysed further.

Motoneurone activation patterns

Embryos from stage 35½–38 (drug-treated or control embryos) were sacrificed by decapitation, and isolated spinal cord–hind-limb preparations were prepared as previously described (Landmesser & O'Donovan, 1984*a*). They were kept in well-oxygenated Tyrode solution at 30 °C for 2–3 h, after which time control embryos become spontaneously active. The bath was continuously superfused with fresh Tyrode solution during this period to wash out the applied drugs.

We characterized motoneurone activation patterns by making e.m.g. recordings with suction electrodes from pairs of hind-limb muscles (usually one flexor and one extensor) following single stimuli to the thoracic or cervical cord. In control embryos this procedure sets off a series of kicks and a series of co-ordinated e.m.g. bursts can be obtained from the muscle (see Fig. 1*A*). We recorded the activation patterns on-line on a Gould Chart Recorder (frequency response d.c. 50 Hz) and on a Vetter Instrumentation recorder, for subsequent analysis.

In the isolated cord preparation (Landmesser & O'Donovan, 1984*a*), each cycle is initiated by a brief synchronous activation of all motoneurones. While this synchronous response is not readily apparent on the time base used to present the e.m.g. recordings, its time of occurrence has been noted by arrowheads on the iliofibularis sequence (Fig. 1*E*, bottom trace) since the e.m.g. response in this muscle normally consists of only a synchronous response. Following the synchronous response, flexor motoneurone pools undergo a long silent or inhibitory period of up to 500 ms (see top traces of Fig. 1*A*, *D* and *E*) whereas extensor pools have relatively shorter inhibitory (approximately 100 ms or less) periods (Fig. 1*A*, bottom trace); the latter are not obvious for most

extensor muscles on the time base used. It has been previously shown that motoneurone pools can be characterized as extensor or flexor based on the length of the inhibitory period and their burst duration (see Landmesser & O'Donovan, 1984*a*, for further details). We therefore determined these parameters for the drug-treated and control embryos in the present study. To present the data more quantitatively, we also constructed histograms, which show the periods in each cycle during which each muscle was active (see Fig. 2 and Landmesser & O'Donovan, 1984*a*, for further details).

In addition, we assessed motoneurone activation patterns, especially in cases where peripheral neuromuscular blockade was pronounced, by recording directly from muscle nerves with tight-fitting polyethylene suction electrodes. Such responses were recorded on tape at $7\frac{1}{2}$ ft./s and were played back at $3\frac{3}{8}$ ft./s onto the chart recorder. Responses were also photographed directly from the oscilloscope screen.

Motoneurone cell counts

Following the recording session, the spinal cord and adjacent dorsal root ganglia were fixed overnight in 2% glutaraldehyde. After rinsing in phosphate buffer, the cords were dehydrated, embedded in paraffin, and sectioned at 10 μm as described previously (Landmesser & O'Donovan, 1984*b*). The sections were stained with Cresyl Violet and motoneurones were counted in every fifth section throughout the lumbosacral enlargement. Our control counts correspond well with other published values (Hamburger, 1975; Pittman & Oppenheim, 1979).

RESULTS

Suppression of embryonic motility and motoneurone cell death by chronic exposure to dTC

As reported by Pittman & Oppenheim (1979), chronic *in ovo* injections of *d*TC greatly reduced spontaneous embryonic motility. We observed that the number of hind-limb movements in treated embryos was reduced from control values of $11{\cdot}7 \pm 3{\cdot}2$/min to $0{\cdot}74 \pm 0{\cdot}9$/min (mean ± S.D.; $n = 75$ for controls, 240 for experimentals) for the ages tested (stage 30–39). Even these movements, which are described below, were not of the normal pattern consisting of sequences of alternating bursts between antagonistic muscles. Instead, they consisted of a single, brief synchronous activation of most muscles. Motility returned toward normal when *d*TC injections were discontinued for 3 or more days, and in four such embryos (tested between stages 36 and 38) the number of movements reached $9{\cdot}7 \pm 1{\cdot}5$/min.

Blockade of spontaneous motility by a variety of neuromuscular blocking agents has been found to prevent normal motoneurone cell death (Pittman & Oppenheim, 1979). During the cell-death period, chick lumbosacral motoneurones are reduced from approximately 20000 to 10000 per side (Hamburger, 1975; Chu-Wang & Oppenheim, 1978). In many of our embryos, chronic exposure to *d*TC blocked almost all of the normal cell death; counts at stages 36–39 (post-cell death) yielded a mean number of 19017 ± 299 (mean ± S.E. of mean, $n = 10$) motoneurones compared to 19245 ± 424 for the controls. Thus exposure to *d*TC at the doses used can greatly reduce normal motility and prevent virtually all motoneurone cell death in some embryos. It is from these embryos that e.m.g. data in the next section is derived.

In other embryos treated with the same *d*TC concentrations and in which motility was reduced to a similar extent ($0{\cdot}8 \pm 1{\cdot}1$ movements/min), motoneurone cell death was only partly prevented (motoneurone number 13571 ± 199, mean ± S.E. of mean, $n = 6$). It is possible that subtle variations in the amount and pattern of motility not detected in the present paradigm accounted for these different results. Alternatively, muscle atrophy was more extreme in some embryos and may have accounted for the enhanced motoneurone death.

In many cases we observed that flexor muscles such as sartorius and anterior iliotibialis exhibited extensive degenerative changes, including replacement of muscle fibres by fatty tissue. As described more fully below, we also found that *in ovo d*TC application directly affects spinal cord circuits resulting in a tonic excitation of flexor motoneurones. It is possible that excessive activation of flexor muscles could have occurred at times when the neuromuscular junction was incompletely blocked. Due to the small size and large input resistance of young muscle fibres, the safety factor for transmission at these junctions is high. Therefore excessive activation of the muscle could result in direct damage with subsequent degeneration of the motoneurones. This explanation is supported by the fact that in several stage 36 embryos, which had received single injections of *d*TC or α-BTX 10 h prior to sacrifice, the sartorius muscles were found to be super-contracted and pulled away from their origin. It is likely that many of these fibres would have degenerated subsequently. Alternatively, flexor motoneurones may have been caused to degenerate by the drug treatment, the muscle atrophy being secondary. Consistent with both explanations is the finding that cell death was often more extensive in spinal segments with a greater proportion of flexor motoneurones (i.e. LS 1–3). In their earlier study with chronic *d*TC injections, Pittman & Oppenheim (1979) also failed to rescue cells in these segments.

In the four animals which were allowed to recover from *d*TC blockade for 3–7 days before sacrifice which in all cases was after the normal cell-death period (in two *d*TC was discontinued beginning at stage 33, in two at stage 36), motoneurone numbers (10285 ± 598, mean $\pm$ S.E. of mean) differed only slightly from control post-cell-death values of 9409 ± 980. A similar finding has been previously reported by Pittman & Oppenheim (1979).

Motoneurone activation patterns in embryos with reduced motility and blockade of motoneurone cell death

In seven embryos in which motoneurone cell death had been completely blocked, we assessed motoneurone activation patterns indirectly by recording e.m.g.s from pairs of muscles in an isolated spinal cord–hind limb preparation. In this preparation, a single stimulus to the thoracic cord activates intrinsic lumbosacral circuits and sets off a series of alternating bursts in pairs of antagonistic muscles (Landmesser & O'Donovan, 1984*a*). Each cycle corresponds to a single kick, and sequences of up to eight kicks are similar to normal bouts of spontaneous movement recorded *in ovo* (Ripley & Provine, 1972; Bekoff, 1976; Landmesser & O'Donovan, 1984*a*). An example of such a record from a stage 36 control embryo (Fig. 1*A*) shows that antagonistic pairs of muscles are activated out of phase; sartorius, a flexor (top trace), exhibits prolonged bursts which are silenced during activation of the extensor caudilioflexorius (bottom trace). Similar control records can be seen in Figs. 4*A* and 6*A*. Each cycle begins with a synchronous response during which all motoneurones are activated (this response is denoted by arrowheads in Fig. 1*E*; see Methods for additional explanation); following this, flexors are silenced for a long period while extensors have much shorter silent periods (Landmesser & O'Donovan, 1984*a*; O'Donovan, 1984). When histograms were constructed to show the probability of a muscle being active at any phase of the cycle, each of a number of thigh muscles was

previously shown to have a relatively unique activation pattern. Flexors could be distinguished from extensors, and within each class several muscles could be unambiguously identified by their activation patterns (Landmesser & O'Donovan, 1984*a*).

When isolated spinal cord–hind limb preparations from *d*TC-treated embryos were washed with avian Tyrode solution, neuromuscular transmission rapidly recovered (see also Ding, Jansen, Laing & Tonnesen, 1983) and within 1 h vigorous muscle contractions could be evoked by electrically stimulating spinal nerves. However, such preparations did not exhibit the spontaneous burst sequences characteristic of control preparations which have been kept in warm Tyrode solution (30° ± 2 °C) for 1–2 h. Stimuli to the thoracic cord also failed to evoke patterned activity. The examples shown in Fig. 1*B*–*F* are representative of these preparations.

During the early stage of washing, flexors such as sartorius tended to have a high level of tonic background activity, whereas extensors such as caudilioflexorius did not. At first no patterned motor activity or bursts could be elicited and stimuli to the thoracic cord caused a variable increase in tonic activity in flexors and often set off a rhythmic series of synchronous responses in extensors (see Fig. 1*B*). After several more hours of washing, patterned activity could be evoked (Fig. 1*C*) although it differed from controls in that sartorius bursts were shorter and exhibited minimal silent or inhibitory periods during extensor activation. Extensor bursts were closer to normal but were significantly shorter than control responses. However, after 6–7 h of washing, the cord was capable of generating patterned bursts that differed only slightly from controls (Fig. 1*D*, *E* and *F*).

The sartorius bursts shown in Fig. 1*D* and *E* were within the control range in duration, although their inhibitory periods were shorter (334 ± 81 ms *vs.* 522 ± 80 ms for controls) (see also Fig. 2). Iliofibularis (Fig. 1*E*, bottom trace) produced only a synchronous response, as it does normally in isolated cord preparations (Landmesser & O'Donovan, 1984*a*). However, some differences were observed in other muscles. In general, many muscles, especially extensors, had shorter bursts than normal. For example, in Fig. 1*F*, both the anterior and posterior iliotibialis had shorter bursts than normal (see also Fig. 2), and shorter inhibitory periods as well.

The activation patterns of the sartorius and caudilioflexorius motoneurone pools from five embryos which had been chronically treated with *d*TC and then washed for 5–7 h are summarized in the histograms of Fig. 2*B*. Sartorius was still activated as a flexor, although both its silent period and burst duration were shorter than those of controls (Fig. 2*A*). Similarly, the caudilioflexorius motoneurones were activated in an extensor pattern, although burst durations were again shorter than controls (compare Fig. 2*A* and *B*).

In general, all of the muscles recorded from (sartorius, caudilioflexorius, anterior iliotibialis, posterior iliotibialis, iliofibularis, external adductor, and femorotibialis) were activated as flexors or extensors based on previously defined criteria (Landmesser & O'Donovan, 1984*a*) and none differed in their classification when compared with controls. In addition, although the inhibitory periods and burst durations were shortened for all muscles, these changes were proportional to those of controls for all muscles. Thus, if muscles were rank-ordered based on the length of either their inhibitory period or burst duration, the rank-ordering did not differ between control and chronically *d*TC-treated embryos.

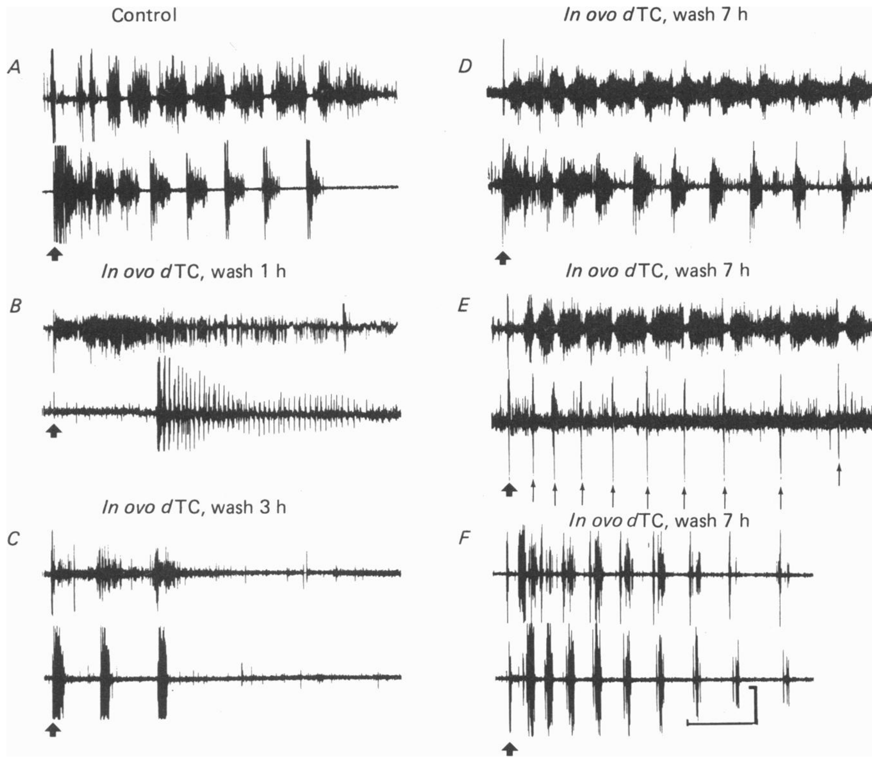

Fig. 1. E.m.g. recordings from embryos chronically treated with *d*TC, *A*, in a movement sequence elicited from a control embryo by a single shock to the thoracic cord (arrow at start of traces) sartorius (flexor), top trace, is activated out of phase with caudilioflexorius (extensor). *B*, e.m.g.s from the same pair of muscles from an isolated cord taken from chronically *d*TC-treated embryo washed for 1 h, at which time neuromuscular transmission had recovered. Sartorius (top trace) failed to exhibit discrete bursts, there being only a tonic increase in activity. Part of the way through the sartorius response, a series of synchronous responses was set off in caudilioflexorius. *C*, after 3 h of washing, the same muscle exhibited discrete bursts, but there was no inhibition of sartorius activity during the caudilioflexorius burst (compare with *B*). *D*, after 7 h of washing, both muscles were activated in a series of alternating bursts that differed only slightly from the control response. *E*, sartorius (top trace) from a second *d*TC-treated embryo also exhibits a normal activation pattern after 7 h of washing. Iliofibularis (bottom trace) exhibits only synchronous responses (indicated by thin arrows) as it does in the control embryo (not shown). *F*, activation patterns of the anterior iliotibialis (top trace) and posterior iliotibialis (bottom trace) from a third *d*TC-treated embryo. Both muscles have shorter bursts than normal but anterior iliotibialis has a relatively long inhibitory period following the synchronous discharge beginning each burst, which is the typical flexor pattern. The posterior iliotibialis has a much shorter inhibitory period, characteristic of a normal extensor. All embryos at stage 36–36½. Calibration bars = 5 s, 2 mV.

The medial adductor shown in Fig. 2*C* illustrates this point. Normally, the adductor has an inhibitory period that is intermediate in length between those of caudilioflexorius and sartorius. The same is true of the *d*TC-treated muscle, even though both its inhibitory period and burst duration are significantly shortened.

In two preparations, in which complete block of motoneurone cell death was later confirmed, sartorius recovered completely with washing, as shown for one embryo

in Fig. 2*D* (compare with Fig. 2*A*). The mean sartorius inhibitory period for these two embryos, 504 ± 82 ms, did not differ significantly from the control value of 522 ± 80 ms.

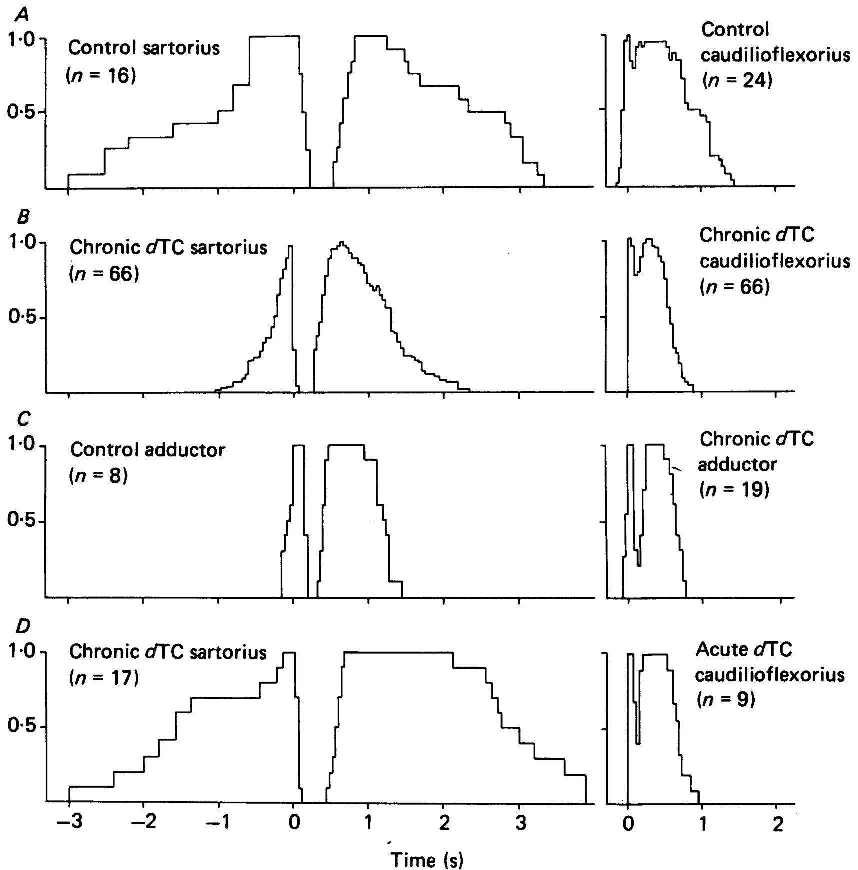

Fig. 2. Histograms displaying the probability of activity during each cycle (time from one synchronous discharge to the next) of various muscles from control and *d*TC-treated embryos following 5–7 h of washing. Histograms were constructed from expanded time-base records in which each e.m.g. response was aligned at the onset of the synchronous response (= 0 time). The responses were then divided into 40 ms bins, and the proportion of time a given muscle was active during each 40 ms interval plotted on the ordinate. Data in *B* was pooled from five *d*TC-treated embryos. Data in *A* and *D* and the control adductor of *C* were from single embryos, whereas the chronic *d*TC-treated adductor shown in *C* was pooled from four embryos.

Thus, it is possible to conclude that blocking both motoneuronal cell death and the patterned activation of muscles does not perturb the normal development of the cord circuits responsible for the basic activation of flexors and extensors. The changes that were observed (i.e. shorter bursts, shorter inhibitory period etc.) are probably due to a failure of the cord to recover from a direct effect of *d*TC. This possibility was tested in the following experiments.

Five stage 36 embryos, in which normal motoneuronal cell death had already taken place, were injected with 2–3 mg of *d*TC 10 h before they were sacrificed. When isolated spinal cord–hind limb preparations were prepared from these embryos, they

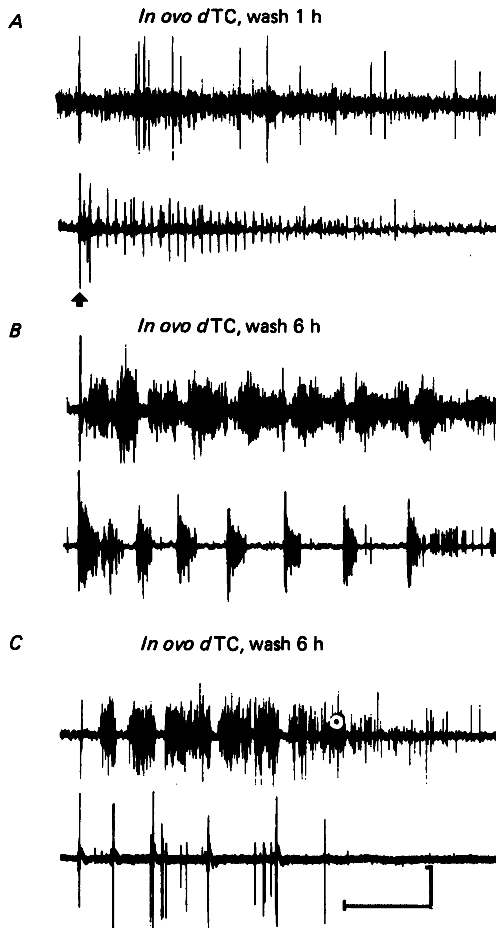

Fig. 3. E.m.g. responses from a spinal cord isolated from a stage 36½ embryo that had received a single *in ovo* injection of *d*TC 10 h before. *A*, after 1 h of washing neither sartorius (top trace) nor caudilioflexorius (bottom trace) exhibit discrete bursts (compare with control and chronic *d*TC-treated muscles in Fig. 1). *B*, after 6 h of washing sartorius (top trace) and caudilioflexorius (bottom trace) exhibit an alternating pattern of activation similar to control. *C*, sartorius (top trace) and anterior iliotibialis (bottom trace) activation pattern from a different embryo (stage 36) which had also received a single *d*TC injection 10 h before and was washed for 6 h. Calibration bars = 5 s, 2 mV for *A* and *B*; 5 s, 1 mV for *C*. Arrow indicates time of stimulus.

exhibited behaviour similar to that of the embryos chronically treated with *d*TC. At first, patterned activity could not be elicited (1 h wash, Fig. 3*A*), but after prolonged washing (4–6 h), alternating bursts between flexors and extensors occurred (Fig. 3*B*). However, as in the chronic *d*TC-treated embryos, there was a tendency for bursts to be shorter, especially in extensors; compare, for example, the activity histogram

for the acutely *d*TC-treated caudilioflexorius (Fig. 2*D*) with the chronic *d*TC and control situations (Fig. 2*B* and *A* respectively). In addition, the inhibitory periods of flexors such as sartorius were shorter than those in controls (300 ± 56 ms *vs.* 522 ± 80 ms), but did not differ significantly from those of the chronic *d*TC-treated animals reported above (334 ± 81 ms). In three of these embryos (see for example Fig. 3*C*, bottom trace), the anterior iliotibialis had very short bursts, similar to the chronically treated embryo shown in Fig. 1*F*.

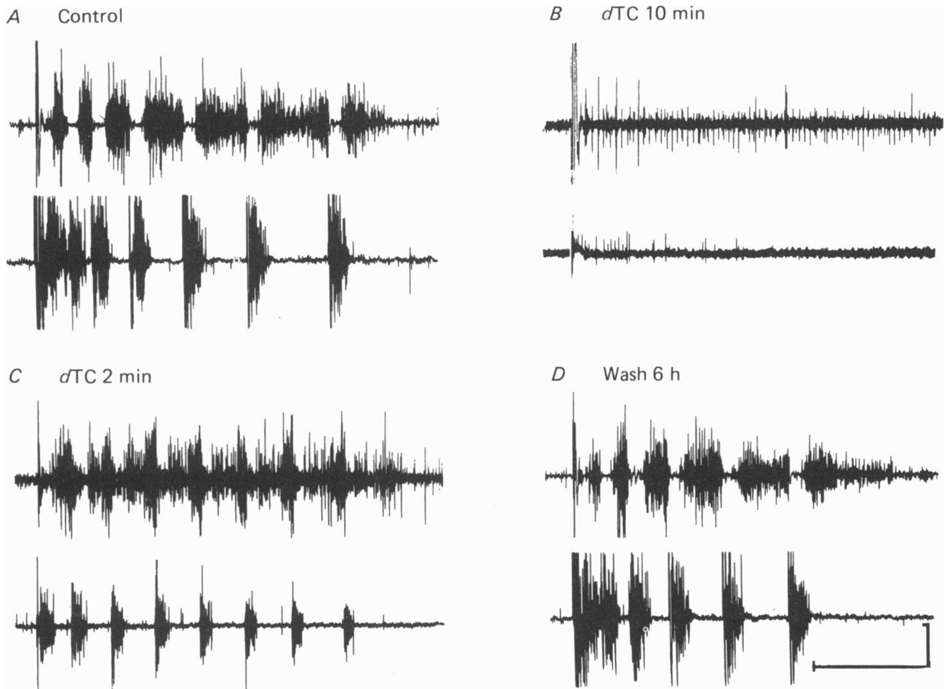

Fig. 4. The effect of bath-applied *d*TC on muscle activation patterns. In all pairs of records sartorius is on the top and caudilioflexorius on the bottom. In the control response (*A*) the two muscles are activated out of phase. Within 2 min of superfusion of the bath with 1×10^{-6} M-*d*TC, the sartorius bursts become less well defined and by 10 min (*C*) no bursting occurs. However, an increase in tonic single-unit activity is still evoked, indicating that the neuromuscular junction has not been completely blocked. Following 6 h of washing the responses (*D*) return to control values. Calibration bars = 5 s, 2 mV.

That *d*TC does have a rapid and direct effect on the cord is shown in Fig. 4. Within 1 min of application of 10^{-6} M-*d*TC to an isolated spinal cord–hind limb preparation, the sartorius bursts became ragged with less-clearly-defined inhibitory periods; the extensor (caudilioflexorius) bursts were depressed in amplitude and shortened. After five additional minutes, stimuli no longer evoked bursts. However, at this drug concentration, neuromuscular synapses were only partly blocked, and direct stimulation of the spinal nerve evoked large synchronous electrical responses from the muscles and vigorous contraction. After a 2–3 h wash, patterned bursts could again be elicited (Fig. 4*D*). It is therefore possible to conclude that a concentration of *d*TC which only partly blocks neuromuscular transmission (the compound e.m.g. response

to direct spinal-nerve stimulation was reduced to approximately two-thirds of normal), completely blocks the patterned activation of motoneurones. Given the similarity between the e.m.g. responses of isolated cords superfused with 10^{-6} M-*d*TC and those subjected to chronic or acute *in ovo* treatment with *d*TC, it seems likely that the *in ovo d*TC dose achieved a similar degree of block.

Although we conclude that the normal patterned activation of muscles, and probably their motoneurones as well, is not required for the development of the cord circuits that produce the alternating activation of extensors and flexors, it took many hours of washing to achieve full recovery of this alternating activation. During this time the cord was intermittently activated, and produced some spontaneous responses as well. Thus, if one assumes that spontaneous activity aids in the refining of cord circuitry, for example by the retraction of synapses, this process could have been operating during the recovery period. Therefore, two isolated cord preparations were prepared from chronic *d*TC-treated embryos which were washed for 6 h at 20 rather than 32 °C. At this temperature, cord circuits are inactive and no spontaneous activity occurred. In addition, no stimuli were applied to the cord. After this period, the first stimulus was seen to evoke normal alternating flexor and extensor activity. The record in Fig. 1*E* is in fact taken from one of these embryos.

We also attempted to subject embryos to treatments that did not affect activation of motoneurones but did block motoneuronal cell death. Weil & Greene (1984) have recently reported that motoneurone cell death can be substantially reduced by treating embryos with dibutyryl-cyclic GMP. Therefore, twenty-seven embryos were chronically treated with dibutyryl-cyclic GMP from stage 28 through the cell-death period. As reported by Weil & Greene (1984), spontaneous activity was not reduced, there being $10{\cdot}4 \pm 5{\cdot}1$ *vs.* $11{\cdot}7 \pm 3{\cdot}2$ kicks/min for controls. We assayed e.m.g. patterns in seven of these embryos at stage $35\frac{1}{2}$–$36\frac{1}{2}$ and found them to be completely normal. In Fig. 5 sartorius shows excellent alternating activity with the extensors posterior iliotibialis (*A*) and caudilioflexorius (*B*). One can also note that another flexor, the anterior iliotibialis (*C*) had shorter inhibitory periods than sartorius, as it does in controls. There were, in fact, no differences between the activation patterns of any of the six different muscles we assayed.

The cords of these embryos were sectioned and the number of motoneurones found to be 13000 ± 320 *vs.* 10220 ± 270 for controls. Motoneurone cell death was therefore only partially reduced by this treatment. However, rescuing these neurones did not affect the activation patterns. This suggests that the death of at least these neurones is not required to develop precise cord circuits capable of appropriately activating motoneurones.

Some controversy exists over the extent to which motoneurone cell death can be prevented by chronic exposure to cyclic GMP (see Oppenheim & Weil, 1985). Cyclic GMP, in our hands and at the concentrations used, had an effect, but resulted in only a moderate rescue of motoneurones. However, we did not pursue these observations further and cannot help in clearing up this controversy.

Finally, we considered using α-BTX, as this has been shown to completely block motoneurone cell death (Pittman & Oppenheim, 1979) and would be expected to be a more specific blocker of peripheral neuromuscular synapses. If true, this would then avoid direct drug effects on the cord, which we assumed were responsible for the minor

changes in pattern which remained after prolonged washing in the embryos chronically exposed to *d*TC. Therefore several isolated spinal cord–hind limb preparations were treated with progressively larger doses of α-BTX up to a concentration of 4 μg/ml. Since α-BTX was expected to completely and irreversibly block the neuromuscular junction, we assayed motoneurone activation patterns by recording from muscle

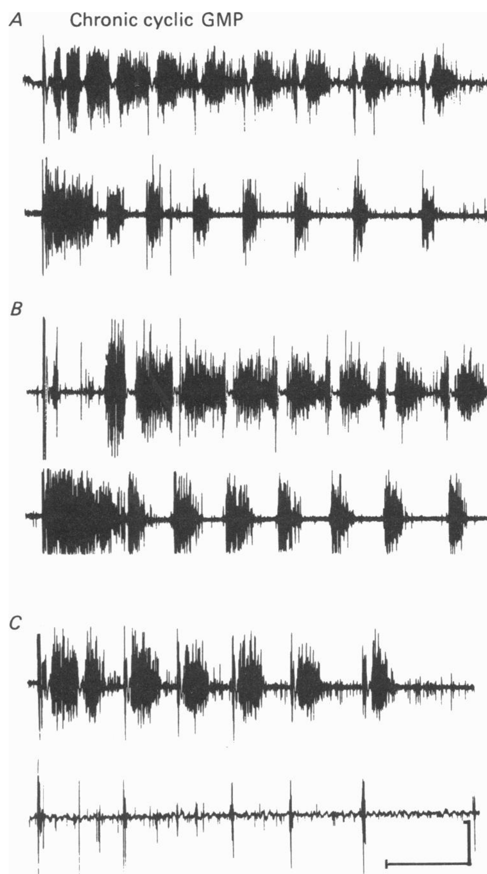

Fig. 5. E.m.g. responses from stage $35\frac{1}{2}$–36 embryos in which motoneurone cell death had been partially blocked by chronic application of dibutryl-cyclic GMP. None of the activation patterns shown differs appreciably from controls. *A*, sartorius (top trace) and posterior iliotibialis (bottom trace). *B*, sartorius (top trace) and caudilioflexorius (bottom trace). *C*, anterior iliotibialis (top trace) and iliofibularis (bottom trace). Calibration bars = 5 s, 2 mV.

nerves with suction electrodes. Fig. 6*A* and *B* show that α-BTX was similar to *d*TC in suppressing the patterned activation of motoneurones. As with *d*TC, this occurred at concentrations (2 and 3 μg/ml) which did not completely block the neuromuscular junction. Vigorous contractions still occurred in muscles with intact nerve supplies. Only at concentrations of 4 μg/ml was muscle contraction completely blocked.

Although α-BTX affects the activation patterns of motoneurones in isolated cord preparations, its larger molecular size (compared to *d*TC) might result in less-complete

access to the spinal cord when applied *in ovo* to the chorioallantoic membrane. Therefore, in two stage $35\frac{1}{2}$ embryos 75 μg of α-BTX was applied to the chorioallantoic membrane. This is the standard dose used to block motoneurone cell death (Pittman & Oppenheim, 1979). The next day, the spontaneous motility of these embryos was

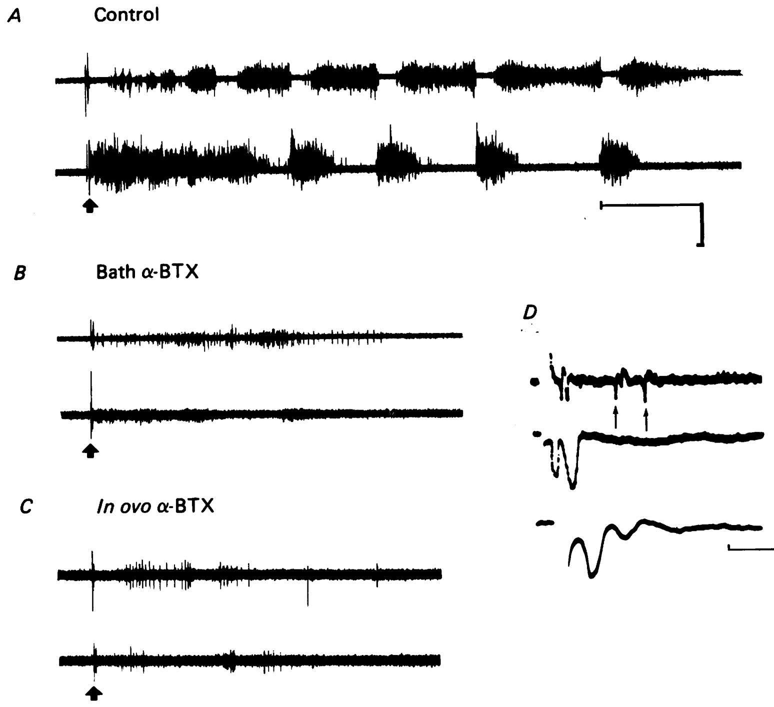

Fig. 6. Motoneurone activation patterns from stage 36 isolated spinal cord preparations following *in ovo* or bath-applied α-BTX. *A*, control neurogram recordings from the sartorius (top trace) and caudilioflexorius (bottom trace) muscle nerves indicate that the two motoneurone pools are activated out of phase. *B*, following bath application of 3 μg/ml α-BTX bursts no longer occur, although some increase in tonic activity occurs. *C*, similar responses were obtained from a spinal cord isolated from a stage $35\frac{1}{2}$ embryo which had received a single 75 μg injection of α-BTX *in ovo* 16 h before. Arrows indicate time of stimulus. Although only weak tonic activity (indicated by thin arrows) could be elicited in sartorius by stimulating the thoracic cord (*D*, top trace) comparable to top trace in *C*, only at much faster time base, motoneurones could be activated by direct stimulation of the lumbosacral cord (*D*, middle trace) or the LS 1 ventral root (*D*, bottom trace). Calibration bar in *A* is 5 s and applies for *B* and *C* as well; 2 mV for *A* and *B*, 1 mV for *C*. In *D*, calibration bar is for top trace 10 ms, 0·05 mV; for middle trace 5 ms, 0·1 mV; and for bottom trace 2 ms and 0·2 mV.

found to be completely blocked (0 movements in 3 min). We removed the embryos from the eggs and dissected them in a bath continuously superperfused with fresh Tyrode solution (to remove any unbound α-BTX). Following exposure of the spinal cord they were kept in warm Tyrode solution for 2–5 h.

Since α-BTX blocked neuromuscular transmission, we assessed motoneurone activation patterns by recording directly from muscle nerves. As shown in Fig. 6*C*,

these cords failed to produce any patterned motor output or bursts, although stimulation of the thoracic cord produced a slight increase in motoneurone firing (this can also be seen in the oscilloscope recording shown in Fig. 6 *D*, top trace). In contrast, direct stimulation of the ventral roots produced large compound action potentials in these muscle nerves (Fig. 6 *D*, bottom trace). Direct stimulation of the lumbar cord (middle trace) produced a dual component response. The first is due to direct activation of the motoneurones and has a latency similar to the response to ventral root stimulation shown in the bottom trace. The second, longer latency response appeared to be synaptic, and was easily blocked with repetitive stimulation. Thus motoneurones can be synaptically activated, although the circuitry responsible for generating bursts appears inactivated.

Cord circuits were only affected by α-BTX at concentrations considerably higher than that needed to block the mature skeletal neuromuscular junction (Bursztajn & Gershon, 1977). Therefore it is possible that these central synapses were actually blocked by other α-toxin contaminants which are known to block chick ciliary ganglion synapses (Berg & Ravdin, 1979; Chiappinelli, Cohen & Zigmond, 1981). Deductions as to the pharmacological nature of these central cholinergic synapses thus requires additional study. It was somewhat surprising that such high concentrations of α-BTX were also required to block the neuromuscular junction in the present study. However, differences in the properties of the developing acetylcholine receptors at these immature synapses could well account for this. Nevertheless, the cord circuitry required for proper alternating activation of muscles was blocked by doses of either *d*TC or α-BTX which others have used to block embryonic motility and motoneurone cell death (Pittman & Oppenheim, 1979) and which we have shown are necessary to completely block neuromuscular junctions when bath-applied. There is therefore a need for caution when interpreting the results of drugs when applied systemically *in ovo*.

DISCUSSION

The major finding of this study is that motoneurones form generally appropriate connexions both peripherally and centrally when naturally occurring motoneurone cell death is completely prevented. This extends the observation of Oppenheim (1981), who showed that following blockade of motoneurone cell death, motoneurones projecting to specific muscles were located in appropriate cord positions. By using an additional criterion for identifying motoneurones (i.e. activation pattern), the present study strengthens the conclusion that motoneurones which have been rescued from cell death are not projecting to inappropriate muscles.

For example, Oppenheim (1981) found that following the prevention of motoneurone cell death, motoneurones projecting to the sartorius muscle were localized in the region of the control sartorius motoneurone pool. However, since motoneurones cannot be classified unambiguously by position alone, the possibility remains that many motoneurones projecting to sartorius were actually foreign motoneurones from immediately adjacent pools which would normally be removed by cell death. Lamb (1976) has proposed that the major role of motoneuronal cell death is to remove neurones that have formed erroneous peripheral connexions (but see Farel & Bemelmans, 1985). However, since the activation patterns of the adjacent moto-

neurone pools differ considerably from that of the sartorius muscle (Landmesser & O'Donovan, 1984*a*) and since it has been shown that such pools retain their characteristic activation patterns even when innervating foreign muscles (Landmesser & O'Donovan, 1984*b*), had any significant number of such motoneurones innervated the sartorius muscle, they should have been detected in the e.m.g. or muscle-nerve recordings. This was not found for any of the muscles studied (flexor or extensor) and therefore provides strong evidence that most motoneurones in the embryos in which cell death had been blocked were projecting to appropriate muscles.

In addition, the characteristic activation pattern of a number of motoneurone pools was not altered in these embryos. This indicates that motoneurone cell death does not remove motoneurones that have formed grossly inappropriate central connexions, of the type that would cause them to be activated as extensors rather than flexors. The implication then is that motoneurone cell death is not involved in the basic process by which flexor and extensor motoneurones become synaptically connected to appropriate pre-motor interneurones (see also Bekoff, 1976).

However, motoneurones might have made more subtle errors in connectivity, in the cord as well as in muscle, which were not detected. For several species it has been reported that topographic projections within muscles are relatively diffuse at first and become more precise some time after initial connexions form (Bennett & Lavidis, 1981; Brown & Booth, 1983; Bennett & Lavidis, 1984). It should be possible to determine if topographic projections within muscles are less precise in embryos in which motoneurone cell death and activity have been blocked by using electrophysiological as well as glycogen-depletion techniques.

The central connexions responsible for activating motoneurones as flexors or extensors in the isolated cord preparation are propriospinal, since motoneurone activation patterns are unaltered by disrupting both descending and afferent sensory input (Landmesser & O'Donovan, 1984*a*). Therefore it is possible that some of the rescued motoneurones had in fact made inappropriate connexions with inputs other than propriospinal, and would have normally been removed during the cell-death period. Additional studies will be necessary to assay the specificity of each of these inputs following blockade of motoneurone cell death.

A novel finding of the present study was that patterned motoneurone activity was blocked by concentrations of cholinergic antagonists necessary to block spontaneous motility. This indicates a need for caution when interpreting the results of systemic drug application in young embryos. It would appear that cholinergic circuits within the chick cord are important for producing the patterned activation of muscles and that even large molecules such as α-BTX are able to gain access to the cord, and prevent their activation. Although no direct recordings were made *in ovo* from motoneurones or muscle nerves, the similarity between the effects of *d*TC chronically or acutely administered *in ovo* and that following direct application to isolated spinal cords, leads to the reasonable inference that the patterned activation of motoneurones was blocked in the drug-treated embryos.

It is generally interpreted (Pittman & Oppenheim, 1979) that blockade of neuromuscular junctions by cholinergic antagonists in some way prevents the normal competitive interactions between motoneurones that culminate in cell death. Based on the present results, one cannot rule out an additional central action of the applied

drugs. However, the original interpretation of the data may still be correct, since Oppenheim has found that *in ovo* injections of γ-aminobutyric acid (GABA) and diazepam both block motility, presumably by an effect on cord circuits, yet have no effect on motoneurone cell death (R. Oppenheim, personal communication). Nevertheless, in future experiments it will be important to assay directly the effect of such systemically applied drugs both on motoneurones and on the circuits of pre-motor interneurones responsible for their activation.

Finally, the unexpected finding that chronic *in ovo* injections of *d*TC or α-BTX probably prevent the patterned activation of motoneurones as well as blocking overt movement, leads us to conclude that neither such patterned activation nor the sensory feed-back from the movements so evoked are needed for motoneurones to connect with appropriate circuits of pre-motor interneurones. This observation then stands in contrast to many observations on developing sensory systems, where activation of neuronal circuits plays an important role in shaping the final pattern of connexions (Stryker, 1981; Meyer, 1982; Boss & Schmidt, 1984; Reh & Constantine-Paton, 1985; Sanes & Constantine-Paton, 1985). However, qualifications of this conclusion, similar to those discussed earlier with respect to cell death, also apply here. For example, it is possible that segmental projections within muscles may be less sharply defined in such preparations. In addition, functional activation of developing cord circuits may be required to allow motoneurones to form their entire set of connexions, including those with appropriate descending and sensory inputs. It is also possible that circuits of pre-motor interneurones were functioning normally in our chronic *d*TC-treated embryos, and that only the final element, the motoneurone, failed to be activated in a patterned manner. A determination of this and an analysis of cords in which more elements of the circuit have been blocked, for example by tetrodotoxin (TTX), will be needed to fully assess the role of function in the development of spinal motor circuits.

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