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MAINTAINED FUNCTION OF FOREIGN AND APPROPRIATE JUNCTIONS ON REINNERVATED GOLDFISH EXTRAOCULAR MUSCLES

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SUMMARY

1. The ability of a multiply innervated muscle to become dually innervated, that is to accept a functional innervation from both its original and a foreign nerve, was investigated using the superior oblique muscle (s.o. muscle) of the goldfish.

2. Dual innervation of s.o. muscles was achieved by allowing the original nerve (cranial NIV) to regenerate to its s.o. muscle which had been previously denervated and cross-innervated by a foreign nerve (cranial NIII), or by allowing the original and the foreign nerve to regenerate simultaneously to a denervated muscle.

3. Behavioural observations suggested that in some fish reinnervation of the s.o. muscle by its original nerve repressed the function of a previously established foreign innervation. However, physiological tests which involved the stimulation of both foreign and appropriate nerves, and the recording of mechanical and electrical activity of the s.o. muscle, demonstrated that there was no functional displacement of foreign innervation on these muscles, even on individual dually innervated fibres.

4. Dual innervation of the s.o. muscle persisted, apparently unchanged, for as long as the observations were continued (up to 7 months). The s.o. muscle contains two populations of fibres, fast and slow, and both types became and remained dually innervated.

5. When both NIII and NIV were allowed to regenerate simultaneously to a denervated s.o. muscle there was no obvious selectivity in the final pattern of innervation. On the average both nerves elicited approximately equal tension from s.o. muscles, and evoked excitatory junctional potentials (e.j.p.s) of similar mean quantal contents.

6. 'Myotypic respecification' was shown not to be responsible for the

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discrepancy between the behavioural results which suggested that repression of foreign innervation had occurred, and the physiological results which demonstrated that this was not the case. Anatomical and physiological findings indicated that the discrepancy was attributable to eye rotation produced by regenerated inferior oblique muscle fibres which contracted simultaneously with the cross-innervated s.o. muscle. The net result was an eye movement in which the activity of the s.o. muscle was masked.

7. It is concluded that repression of established foreign neuromuscular connexions following reinnervation by the embryologically correct nerve does not occur on goldfish extraocular muscles. The s.o. muscle can become non-selectively innervated by both foreign and appropriate axons, and remains so, at least for several months.

INTRODUCTION

When peripheral motor nerves are surgically crossed or are cut and allowed to regenerate, the extent to which normal co-ordinated movement is restored is variable. The recovery of apparently normal function following nerve transection in fish (Sperry, 1950; Sperry & Deupree, 1956; Sperry & Arora, 1965; Mark, 1965) and following nerve crosses in urodeles (Grimm, 1971; Cass & Mark, 1975) contrasts with the situation in mature anuran tadpoles (Sperry, 1947) and mammals (Sperry, 1941), which fail to re-establish co-ordinated movements. Of special interest are the findings on goldfish recovering from various experimental procedures in which extraocular muscles became reinnervated by foreign nerves. Behavioural observations suggested that the embryologically correct nerve, given the opportunity to regenerate, could regain exclusive control over the muscle it originally supplied (Sperry & Arora, 1965; Marotte & Mark, 1970*a*; Mark, Marotte & Mart, 1972). These results together with the finding of normal ultrastructure of all neuromuscular junctions examined (Marotte & Mark 1970*b*; Mark *et al.* 1972) were taken to indicate that functional suppression of foreign innervation had occurred as a consequence of the restoration of the correct innervation. It was further suggested that the recovery of co-ordinated movements after nerve transection in lower vertebrates was due to a selective reinnervation brought about by an initial random reinnervation of muscles, followed by a similar type of repression of mismatched connexions (Mark, 1974). The mechanism of repression of neuromuscular junctions was proposed as one which can operate only on fibres with multiterminal, polyneuronal innervation characteristic of most lower vertebrate muscle and not on focally innervated fibres characteristic of most mammalian and anuran muscle. The latter type of fibre therefore becomes randomly reinnervated and remains so.

However, behavioural observations do not always give an adequate indication of what is happening at the level of the neuromuscular connexions. Movements produced by incorrect nerve-muscle combinations could be obscured by those due to some appropriately reinnervated musculature (e.g. Sperry, 1950; Czéh & Szekely, 1971); alternatively, the central connexions of foreign motoneurones could undergo 'myotypic respecification' (that is, reorganize to a pattern appropriate for the embryologically correct motoneurones (Weiss, 1936; Sperry, 1941)). The experiments on goldfish eye muscles presented in this report were designed to examine the physiological function of neuromuscular connexions at a time when competitive reinnervation produced behavioural signs of an apparent selective reinnervation and/or synaptic repression. The results show that in this preparation neither physiological repression of foreign innervation nor selective reinnervation occurs. Individual fibres in the eye muscle became and remained innervated by both foreign and embryologically appropriate nerves. In addition, myotypic respecification was shown not to be responsible for the observed behaviour. Some of the results of the present investigation have been published in a preliminary report (Scott, 1975).

METHODS

The preparation. The superior oblique (s.o.) and inferior oblique (i.o.) extraocular muscles have anatomically separate nerve supplies, being innervated by the trochlear (NIV) and a branch of the oculomotor (NIII) cranial nerves, respectively. This allows electrical stimulation of an identified population of axons entirely appropriate or entirely foreign to the s.o. muscle. In the goldfish the two oblique muscles, when reflexly activated, rotate the eye in opposite directions (Inset in Text-fig. 2). This reflex response provides a behavioural indicator of functional innervation and muscle contraction. The function of correct and inappropriate neuromuscular combinations could therefore be assessed both behaviourally and physiologically in the same animal.

Surgical procedures. Goldfish (*Carassius auratus*), 7–12 cm long, were anaesthetized with ethyl *m*-aminobenzoate methanesulphonate (MS-222, Eastman, 0·08–0·12 g/l.) and wrapped in wet gauze. In one group of fish (Dual-Stag) NIV was cut as it entered the orbit, the i.o. muscle was removed, and the i.o. branch of NIII was sutured to the distal stump of NIV near the s.o. muscle. With this procedure NIII had a shorter distance to grow to reach the s.o. muscle than did NIV, and functional dual innervation by NIII and NIV occurred in forty-eight of the sixty-six fish examined. Most fish were reoperated 14–28 days after the initial surgery to remove any regenerated i.o. muscle fibres.

In another group of fish (Dual-Sim) NIII and NIV were encouraged to regenerate simultaneously to the s.o. muscle. In these animals NIV and the i.o. branch of NIII were cut near their respective muscles and the proximal stumps were sutured together. Dual innervation was accomplished in twenty-three of the twenty-four s.o. muscles operated in this way. Operated animals were individually identified by a coloured thread tied through the mouth and operculum.

Behavioural observations. The i.o. and s.o. muscles are antagonists which mediate a reflex ocular counterrotation tending to keep the eye horizontal when fish are tipped head down or head up (Marotte & Mark, 1970*a*). Reflexes were tested every 3 days in

fifty-nine animals in the Dual-Stag group for as long as 5 months after surgery. Fish were tilted head up or down in a testing apparatus (Traill & Mark, 1970), and the deviation of the body and eye from the horizontal was measured to the nearest degree in several positions. When several measurements of the deviation were made at any selected body angle they were always found to differ by only 2 or 3 degrees; consequently during routine testing it was sufficient to make only one measurement at each body angle. The difference between eye angle and body angle is the 'ocular rotation'; this was plotted as a function of body position. The representative ocular rotation curves from one fish shown in Text-fig. 2 indicate the extent of eye rotation produced by a normal unoperated fish, and the amount of variability among measurements taken from one animal on different days. The changes in ocular rotation which occurred in operated animals were small, and occasionally it was difficult to classify a fish unambiguously as 'repressed', cross-innervated, or reinnervated. However, the behavioural observations provided a means for selecting fish for physiological experiments.

Physiological investigations of neuromuscular function. Experimental animals were killed 3–30 weeks following surgery. Their heads were placed in a bath containing oxygenated (95% O_2, 5% CO_2) Ringer solution of the following composition: 122 mM-NaCl, 2·5 mM-KCl, 1·8 mM-$CaCl_2$, and 17 mM-$NaHCO_3$. The s.o. and i.o. muscles were partially dissected, leaving intact their attachments to the body orbit and to a small piece of sclera. The intracranial portions of NIII and NIV were exposed and stimulated with suction electrodes.

In some animals contraction of the s.o. muscle was observed only visually. In most isometric tetanic tension in response to indirect stimulation (500 msec train of 0·01–0·05 msec pulses, 200–350/sec) was measured with a Harvard Apparatus model 363 force transducer (sensitivity, 0–10 g, 200 mV/g) and recorded with a Gould Brush 220 recorder. Quantitatively equivalent results were obtained with two other transducers which were used in a few experiments. In some cases tension was expressed as kg/cm^2, the cross-sectional area of the s.o. muscle being measured from photographs of histological sections (see Histology below) taken from approximately the same level in all muscles. These techniques may over-estimate slightly the tension/cross-sectional area, for some fibre shrinkage may have occurred during fixation.

Compound junctional potentials and spikes were recorded extracellularly from groups of two to five muscle fibres with suction electrodes (of approximately 100 μm tip diameters) applied directly to the surface of the muscle, and were amplified with a Grass P-15 a.c. preamplifier. Synaptic potentials were recorded intracellularly from single fibres with micro-electrodes (3 M-KCl filled glass micropipettes) of 40–100 MΩ resistances attached to a WPI M-4A probe and d.c. amplifier. Synaptic potentials, recorded both extracellularly and intracellularly, were displayed on a Tektronix R5030 oscilloscope and photographed with a Grass C4 camera. To facilitate intracellular recording the dense layer of connective tissue covering the s.o. muscle was removed by treating preparations with collagenase (20 u./ml.) for 20 min, and then rinsing thoroughly. Preparations in which single nerve stimuli evoked vigorous twitches were treated with a modified Ringer solution containing 1·5–6·5 mM-$MgCl_2$ and 0·5 mM-$CaCl_2$ in order to reduce the number of fibres contracting.

Histology. Most experimental muscles were fixed in 10% formalin and post-fixed in 2% OsO_4. Frozen sections were cut and stained with a saturated solution of Sudan Black B. In other muscles acetylcholinesterase was localized at neuromuscular junctions with a modified Koelle technique (Naik, 1963).

RESULTS

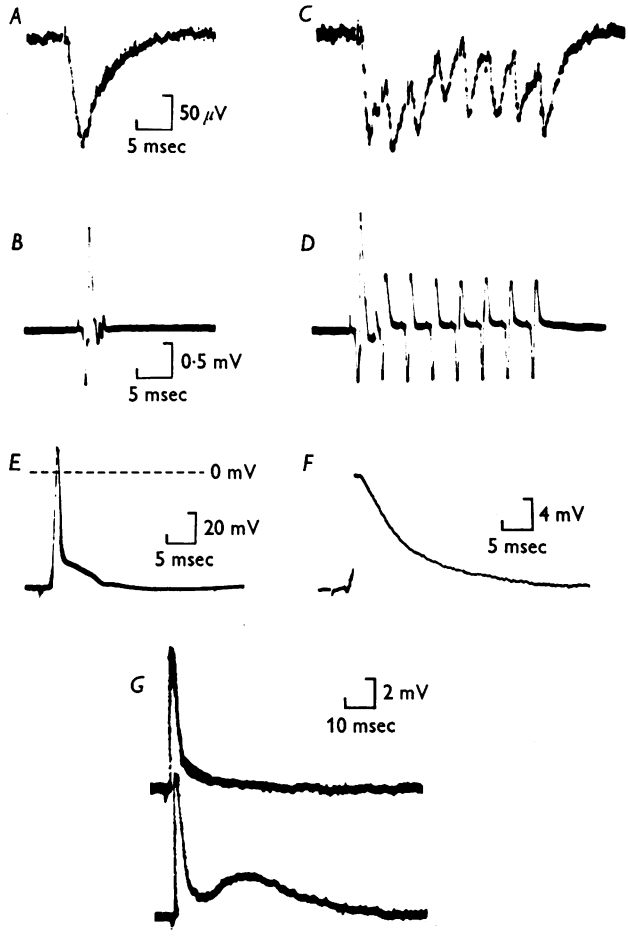
Activity of normally innervated s.o. muscles

The superior oblique (s.o.) and inferior oblique (i.o.) muscles, innervated respectively by the trochlear (NIV) and a branch of the oculomotor (NIII) cranial nerves, are each composed of two anatomically distinct types of multiply innervated fibres (Davey, Mark, Marotte & Proske, 1975). This difference in the morphology of end-plates on fast and slow s.o. muscle fibres was confirmed in the present study (Pl 1 *B*, *C*). In addition, recorded junctional activity revealed that the two fibre types differed physiologically. Each fibre type is located in a discrete region of the muscle (Kilarski & Bigaj, 1969); compound junctional potentials could therefore be recorded extracellularly from small groups of exclusively one fibre type by placing a suction electrode on the region of fast or slow fibres on the orbital (outer) surface of the s.o. muscle (Pl. 1 *A*). Stimulation of NIV elicited small, slow, graded excitatory junctional potentials (e.j.p.s) in slow fibres (Text-fig. 1 *A*) and all-or-none action potentials or rapid e.j.p.s in fast fibres (Text-fig. 1 *B*).

Junctional activity recorded intracellularly from single fast fibres was consistent with the extracellular records from groups of fibres; individual fast fibres responded to nerve stimulation with either overshooting action potentials (Text-fig. 1 *E*) or e.j.p.s (Text-fig. 1 *F*, *G*). Multicomponent e.j.p.s were recorded in 18% of the 318 fibres sampled, ranging from 0 to 53% in thirteen s.o. muscles. In one-third of the fibres which did have these complex e.j.p.s, the various components had different thresholds and time courses, as shown in Text-fig. 1 *G*, indicating that fast fibres in the s.o. muscle receive discrete, distributed innervation from several axons. Fibres which did not show composite e.j.p.s may have also received a polyneuronal innervation, with some junctional potentials outside the recording distance of the electrode, since the space constant of the fast fibres was estimated to be 1·3 mm (Scott, 1976) and the fibres were 4–7 mm long. It is unlikely that intracellular recordings were made from slow fibres because their diameters are small (3–5 μm), and very few of them are located on the global (inner) surface which was sampled with microelectrodes (see Pl. 1 *A*).

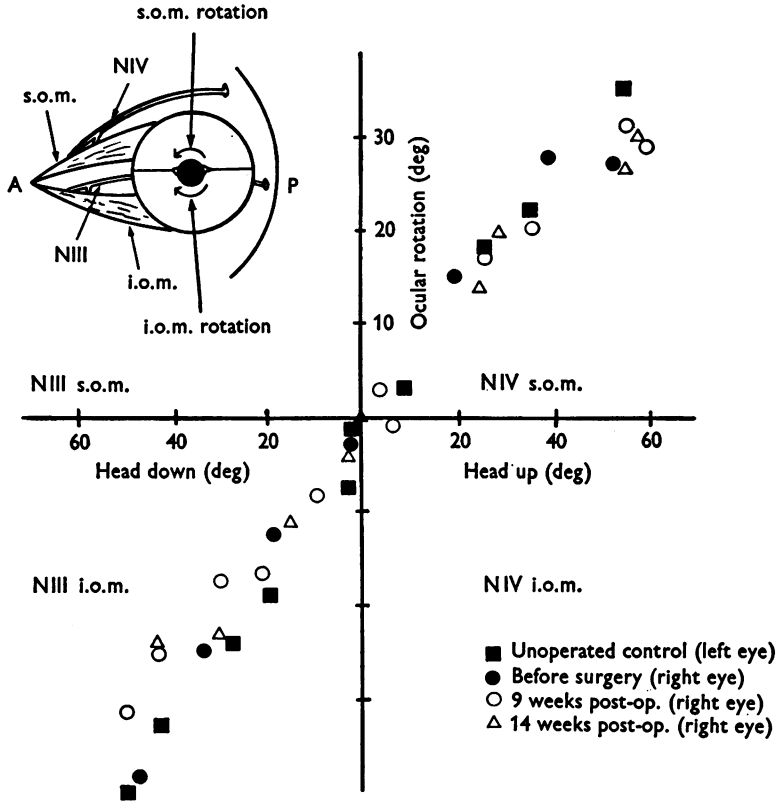
The s.o. muscle produces maximum isometric tetanic tension when NIV is stimulated at frequencies of 200–350/sec. As shown in Text-fig. 1 *C* and *D* junctions on both types of fibres can be repetitively activated at these frequencies, although action potentials fail in fast fibres during such high frequency stimulation. Within this stimulus frequency range, slow fibres contribute only about 15% to the total tension (Scott, 1976).

Reflexly evoked contraction of the two oblique muscles normally acts



Text-fig. 1. Activity of unoperated s.o. muscles recorded in normal Ringer solution. *A–D*, junctional activity recorded extracellularly in response to stimulation of NIV. Negative deflexions are downward. *A*, compound junctional potentials from a small group of slow fibres; the response could be graded in 4–5 steps as stimulus intensity was increased from zero to supramaximal, which is the one shown. *B*, junctional potentials recorded from a small group of fast fibres; there are at least two discrete units of activity comprising the large diphasic spike, probably representing action potentials in two or more fibres. *C* and *D*, responses recorded at the same electrode sites as in *A* and *B*, respectively, to a train of stimuli delivered at 250/sec. The action potentials in fast fibres failed after the first stimulus, but the tension was maximal throughout. *E–G*, intracellular records from single fast fibres. *E*, an overshooting action potential. *F*, a single component e.j.p. *G*, a multi-component e.j.p. The stimulus intensity was increased between the upper and lower traces, bringing in a second component in the latter from a more distant junction. Traces *B*, *D*, and *E* retouched.

to keep the eye horizontal when fish are tilted. NIV fires when fish are tipped head up, activating the s.o. muscle which rotates the eye downward. NIII fires when fish are tipped head down, driving the i.o. muscle which rotates the eye upward. The difference between the orientation of



Text-fig. 2. Ocular rotation measured in one fish, before surgery in which NIV and NIII were cut and the i.o. muscle removed (■, ●) and at two post-operative times after NIV had reinnervated the s.o. muscle and the i.o. muscle had regenerated (○, △). The fish was tilted in various positions (represented on the abscissa), the orientation of the eye measured in each position, and the differences between the orientation of the body and eye with respect to horizontal (ocular rotation, represented on the ordinate) calculated and plotted. The quadrants in which the data points lie indicate which nerve(s) is innervating each oblique muscle, as is indicated for each quadrant. Inset is a diagrammatic representation of the innervation of the s.o. and i.o. muscle. A indicates the anterior (head) end, and P the posterior. Arrows show the direction of eye rotation produced by contraction of each oblique muscle. In head up positions NIV fires, activating the s.o. muscle which rotates the eye downward toward horizontal. In head down positions NIII fires, driving the i.o. muscle which rotates the eye upward toward horizontal.

the eye and body (the ocular rotation, represented on the ordinate) is shown in Text-fig. 2 for a normal unoperated fish held in various positions (represented on the abscissa), and for the same fish after nerve section and regeneration. In this and similar figures eye rotation driven by activity in NIII is represented by data points which lie in the two left quadrants, that due to activity in NIV is represented by points in the two right quadrants; rotation produced by contraction of the s.o. muscle is represented by points in the two upper quadrants, while that due to the i.o. muscle by points in the two lower quadrants. By observing the behaviour and plotting the ocular rotation one can obtain an idea of which nerve(s) innervates each muscle. Normal activation of the s.o. muscle by NIV is indicated by symbols in the upper right quadrant labelled NIV-s.o.m., and normal activation of the i.o. muscle by NIII by symbols in the lower left quadrant labelled NIII-i.o.m.

Activity in dually innervated s.o. muscles

Functional innervation of the s.o. muscle by both NIII and NIV (dual innervation) was accomplished as described in Methods by allowing NIV to regenerate to a cross-innervated s.o. muscle, or by allowing both NIII and NIV to regenerate simultaneously.

Evidence of dual innervation

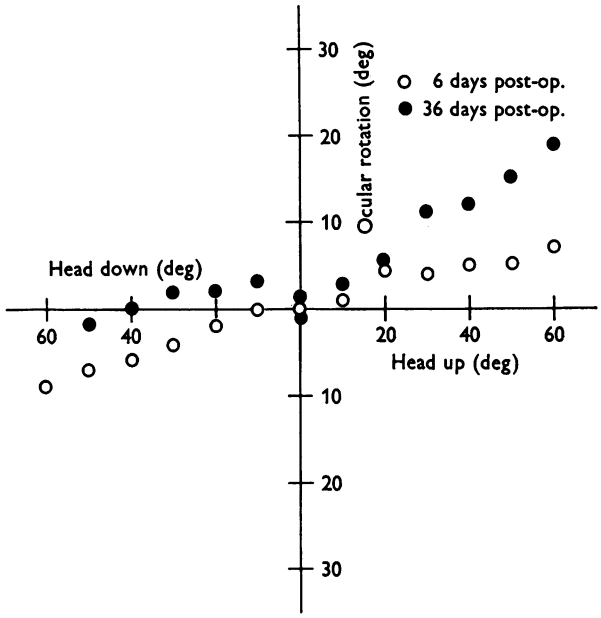
(i) *Behavioural observations.* Seven of the fifty-nine fish tested (Dual-Stag group) exhibited behaviour which suggested that both NIII and NIV innervated the s.o. muscle.

The experimental surgery did not completely abolish reflex ocular rotation. Five to ten degrees of rotation in the normal directions which was produced by the four intact rectus extraocular muscles (Scott, 1976) was always observed following denervation of both oblique muscles. This can be seen by comparing the small amount of ocular rotation observed 6 days after surgery (Text-fig. 3, open circles) with the marked rotation observed normally (Text-fig. 2). Changes in eye movement caused by reinnervation or cross-innervation of the oblique muscles were superimposed on this residual rotation.

When NIII cross-innervates the s.o. muscle, the latter contracts reflexly in head down positions, rotating the eye *downward*, away from horizontal; ocular rotation points representing this reversed eye movement fall in the upper left quadrant (e.g. Text-fig. 3, day 36, and Text-fig. 5, day 23). As in previous investigations (Marotte & Mark, 1970*a*; Mark *et al.* 1972) reversed eye rotation produced by cross-innervated s.o. muscles was never as pronounced as that produced by normally innervated muscles. When dual innervation is achieved both NIII and NIV innervate the s.o. muscle and both reversed eye rotation (in head down positions) and normal eye rotation (in head up positions) should be observed.

Further quantitation of behavioural results was not performed since the main goal of this research was to investigate the physiological responses of operated muscles. The changes in eye rotation in operated animals were small and occasionally it was difficult to classify the s.o. muscle of a fish unambiguously as cross-innervated,

reinnervated or 'repressed'. In addition, as described below, the observed behaviour was often misleading, and in many fish it did not accurately indicate which nerve(s) innervated the s.o. muscle. Even in those animals in which s.o. muscles were re-innervated by NIV alone, the tension produced by that muscle was not correlated with the amount of eye rotation (unpublished observations).



Text-fig. 3. Ocular rotation curve suggesting dual innervation of the s.o. muscle by both foreign (NIII) and appropriate (NIV) nerves. In this fish NIV and the i.o. branch of NIII were cut, the i.o. muscle removed and NIII crossed to the s.o. muscle. Six days after surgery only residual rotation produced by the rectus muscles was observed (○). Thirty-six days after surgery both reversed rotation (● upper left quadrant) in head down positions and normal downward rotation (● upper right quadrant) in head up positions were observed, indicating that both NIII and NIV innervated the s.o. muscles. Axes as in Text-fig. 2.

An example of behavioural records from one of the seven fish in which dual innervation of the s.o. muscle was indicated is shown in Text-fig. 3. In this fish 6 days after surgery neither cut nerve had regenerated, and only residual rotation produced by the rectus muscles was observed (open circles). Thirty-six days after surgery it appeared that both NIII and NIV were driving the s.o. muscle. The upward rotation produced by the rectus muscles in head down positions was overriden by a stronger downward eye rotation, yielding a net reversed rotation (filled circles, upper left quadrant); that is, by day 36 it appeared that NIII was driving the s.o. muscle. In addition, in head up positions normal downward rotation

(filled circles, upper right quadrant) was observed, indicating that NIV also activated the s.o. muscle.

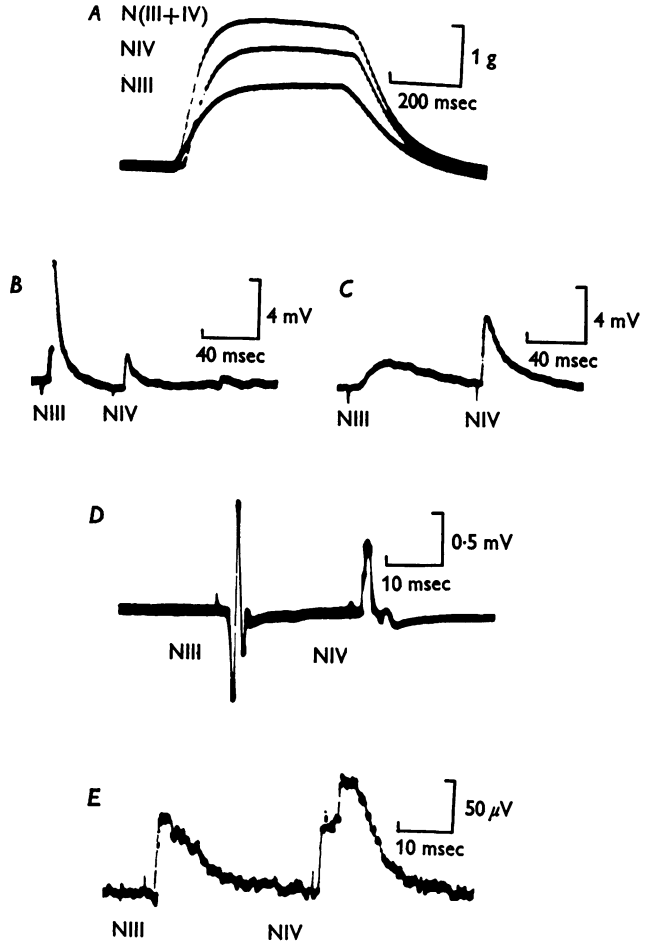
(ii) *Tension occlusion experiments.* More convincing evidence of dual innervation was obtained by electrical stimulation of both NIII and NIV. In seventy-one operated fish (including fish in both Dual-Stag and Dual-Sim groups, see Methods) stimulation of each nerve separately evoked contraction of the s.o. muscle. To estimate the extent to which individual fibres had received dual innervation, the sum of the isometric tetanic tensions evoked by separate stimulation of NIII and NIV was compared with the tension evoked by simultaneous stimulation of both nerves together. Nearly all muscles showed some occlusion of tension. For example in Text-fig. 4*A* the sum of tension produced by the s.o. muscle in response to separate stimulation of NIII and NIV was 37% greater than that produced by simultaneous stimulation of the two nerves. Taking all the animals investigated, the sum of the tension produced by stimulation of each nerve separately was 19·3% ± 14·3 (mean ± S.D., $n = 55$) greater than the tension produced by simultaneously stimulating both. However, in multiply innervated fibres which can contract maximally in the absence of propagated all-or-none spikes, this type of occlusion experiment estimates only the minimum amount of dual innervation (Scott, 1976). These results indicate therefore that on the average at least 20% of the fibres contributing to the generation of tension (largely fast fibres) received innervation from both nerves.

(iii) *Single fibre recording.* Junctional potentials were recorded intracellularly from 435 fibres on the global surface (i.e. from fast fibres) of experimental s.o. muscles 23 days to 7 months after surgery. E.j.p.s evoked by stimulation of both NIII and NIV were recorded at the same electrode site in 20% of the fibres sampled, ranging from 0 to 66% in twenty-one s.o. muscles; examples are shown in Text-fig. 4*B* and *C*. Thus single fibres could indeed become dually innervated, with the foreign and appropriate endings being electrically close.

(iv) *Fast and slow fibres.* Both tetanic tension measurements and intracellular recording sample primarily the population of fast fibres which clearly can be innervated by both foreign and appropriate axons. Extracellular recording of junctional activity from each of four preparations (52–83 days after surgery) demonstrated that slow fibres also received a functional dual innervation. Text-fig. 4*D* and *E* show extracellular records from a typical dually innervated s.o. muscle (52 days after surgery) in which stimulation of both foreign and appropriate nerves evoked junctional potentials in both fast (Text-fig. 4*D*) and slow (Text-fig. 4*E*) fibres.

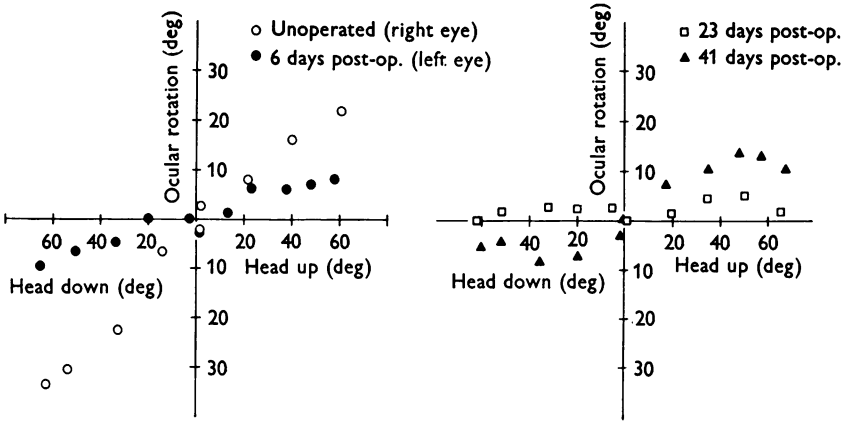
Maintenance of functional dual innervation

Although dual innervation of both populations of muscle fibres in the s.o. muscles was readily achieved, as shown above, it was expected from the work of Mark and collaborators (Marotte & Mark, 1970*a*; Mark *et al.*



Text-fig. 4. Activity in dually innervated s.o. muscles. *A*, isometric tetanic tension produced by stimulation of NIII and NIV separately and then simultaneously [N(III+IV)] 52 days after surgery. There is occlusion of tension, showing that many of the muscle fibres were shared by both nerves. *B* and *C*, intracellular records from two fast fibres 81 and 42 days after surgery, respectively. E.j.p.s. were evoked in both single fibres by stimulation of both NIII and NIV. *D* and *E*, extracellularly recorded compound junctional potentials evoked by stimulation of NIII and NIV from small groups of fast (*D*) and of slow (*E*) fibres 52 days after surgery. Trace *D* retouched.

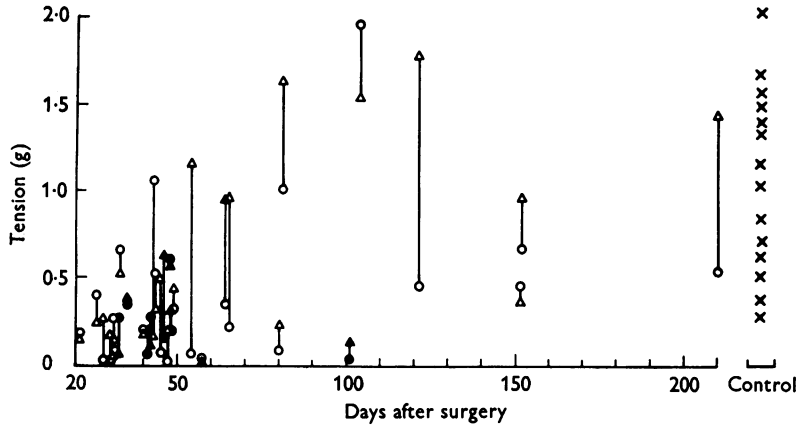
1972) that the function of the foreign neuromuscular junctions would become rapidly suppressed, or at least be reduced in some way. The observed behaviour suggested that such a phenomenon might indeed have occurred in thirteen of the fifty-nine fish tested. In these fish reversed eye rotation in head down positions was observed within 19–26 days after surgery, indicating that the s.o. muscle had become innervated by NIII. Between 22 and 45 days after surgery however this reversed rotation disappeared, and during the following 2 weeks some normal rotation in head up positions returned. For example, Text-fig. 5 shows four ocular



Text-fig. 5. Behavioural findings suggesting repression of foreign (NIII) innervation on the s.o. muscle. In this fish NIV and the i.o. branch of NIII were cut, the i.o. muscle was removed, and NIII was crossed to the s.o. muscle. Six days later only ocular rotation produced by the rectus muscles was observed. On day 23 reversed eye movement was observed in head down positions (□, upper left quadrant), suggesting that NIII had cross-innervated the s.o. muscle, while in head up positions (□, upper right quadrant) rotation was the same as on day 6. By day 41 reversed rotation was no longer observed in head down positions (▲ lower left quadrant), and some normal rotation in head up positions (▲ upper right quadrant) had returned, indicating that now NIV alone innervated the s.o. muscle. These findings could be taken to indicate that junctions from the foreign (NIII) had been repressed by the regenerating appropriate nerve. Axes as in Text-fig. 2.

rotation curves from a fish in which NIV and the i.o. branch of NIII had been cut, the i.o. muscle removed and NIII crossed to the s.o. muscle. Six days later neither nerve had regenerated, and the residual ocular rotation observed (filled circles) was produced by the four rectus muscles. On day 23 reversed eye movement was observed when the fish was held in head down positions (open squares, upper left quadrant), suggesting that NIII had cross-innervated the s.o. muscle; in head up

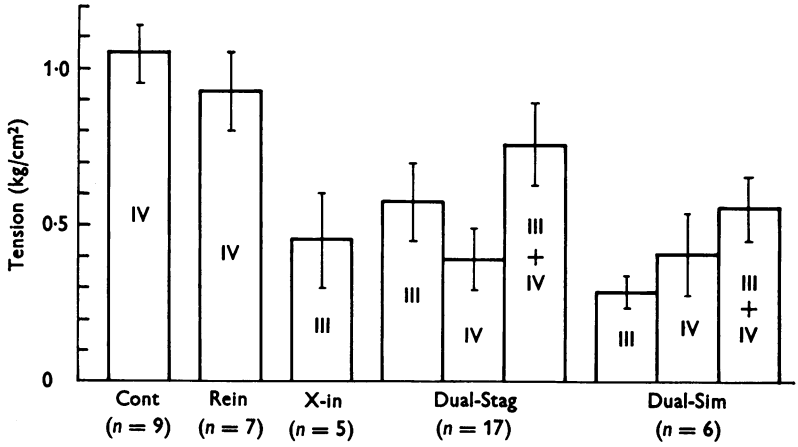
positions (open squares, upper right quadrant) rotation was the same as on day 6, indicating that NIV had not yet regenerated. By day 41 reversed (downward) rotation was no longer observed in head down positions, but a small upward rotation (filled triangles, lower left quadrant) similar to that produced by the rectus muscles on day 6 was recorded, suggesting that junctions from NIII on the s.o. muscle had ceased to function. In addition, some normal rotation in head up positions (filled triangles, upper right quadrant) had returned, indicating that NIV had partially reinnervated the s.o. muscle. If behavioural evidence of this sort genuinely reveals the function of relevant neuromuscular connexions, then these observations indicate that in these thirteen fish reinnervation by the embryologically correct nerve had repressed the foreign junctions.



Text-fig. 6. Isometric tetanic tension produced by dually innervated s.o. muscles in fish killed at various times after surgery. The two tension measurements for each muscle, that due to NIII (△) and that due to NIV (○) stimulation, are connected by a vertical line. Filled symbols represent data from fish in which foreign (NIII) junctions had been judged as repressed by behavioural criteria. Much of the scatter, at least after 40 days, which was sufficient time for complete reinnervation to be achieved, is due to the size range of fish used, as can be seen by comparing experimental values (△, ○) with those of control fish from a similar size range shown on the right (×).

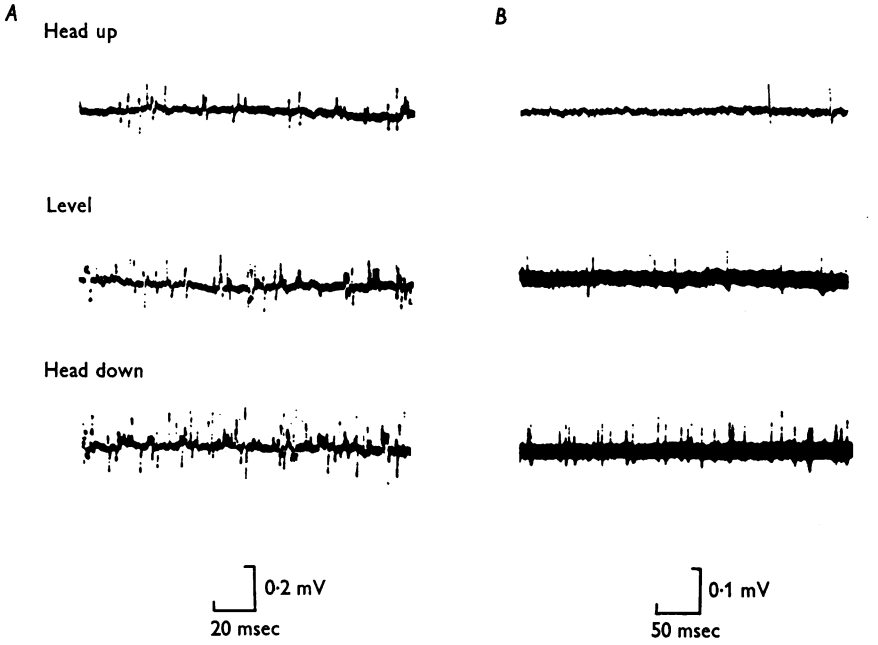
In order to assess critically the validity of the behavioural observations the foreign and appropriate nerves were stimulated and the contraction of the s.o. muscle recorded. In eleven of the fifty-nine fish tested (22–101 days after surgery) junctions from NIII had been judged repressed by behavioural criteria. In all of these stimulation of NIII evoked s.o. muscle contraction, and in ten of the eleven *both* nerves drove the s.o. muscle.

There was no evidence that reinnervation by NIV reduced the function of innervation from NIII. In those fish with apparently repressed foreign (NIII) junctions the average tetanic tensions evoked by stimulation of NIII ($0{\cdot}70 \pm 0{\cdot}48$ kg/cm^2, mean $\pm$ S.D., $n = 7$) and NIV ($0{\cdot}51 \pm 0{\cdot}52$ kg/cm^2, mean $\pm$ S.D., $n = 7$) were similar to the respective tensions evoked by the two nerves in other dually innervated s.o. muscles in fish which had shown no signs of repression ($0{\cdot}53 \pm 0{\cdot}50$ kg/cm^2 and $0{\cdot}31 \pm 0{\cdot}27$ kg/cm^2, mean $\pm$ S.D., $n = 9$). In addition, Text-fig. 6 shows that the relative tension generated by individual dually innervated s.o. muscles in response to stimulation of NIII and NIV was not correlated with the time after surgery, as would be expected if reinnervation by NIV eventually suppressed functional innervation from NIII. Indeed, the tension evoked by



Text-fig. 7. Average isometric tetanic tension ($\pm$ S.E. of mean) generated by s.o. muscles in response to stimulation of NIII and/or NIV in unoperated control fish (Cont) and in various groups of experimental fish. In one group (Rein; measured 21–107 days after surgery) NIV alone had regenerated to the s.o. muscle, in one group (X-in; 26–79 days after surgery) NIII alone had cross-innervated the s.o. muscle, and in the other two groups both NIII and NIV innervated the s.o. muscle. Dual innervation was achieved by allowing NIV to regenerate to cross-innervated s.o. muscles (Dual-Stag; 26–154 days after surgery) or by allowing both NIII and NIV to regenerate simultaneously (Dual-Sim; 33–46 days after surgery), as described in Methods. The nerve(s) stimulated is indicated in each column of the histogram. The average tension evoked from s.o. muscles reinnervated only by NIV (Rein) and the average total tension evoked by simultaneous stimulation of NIII + NIV in both groups of dually innervated s.o. muscles were not significantly different (Wilcoxon test). Nor were there any significant differences among the average tensions evoked by stimulation of NIII in various groups of animals, or between values for NIII and NIV in either dually innervated group.

stimulation of NIII was frequently as great as or even greater than that evoked by stimulation of NIV as long as seven months after surgery. Finally, as shown in Text-fig. 7 the average isometric tetanic tension evoked by stimulation of NIII was approximately the same for s.o. muscles innervated only by NIII and for muscles innervated by both NIII and NIV (compare X-in with Dual-Stag and Dual-Sim). Note, however, that NIII alone innervated the s.o. muscle less effectively and elicited significantly less ($P < 0{\cdot}05$, Wilcoxon test) tension than normal (compare Cont and X-in), while NIV alone could reinnervate the s.o. muscle completely and elicit normal tension (compare Cont with Rein).



Text-fig. 8. Reflexly evoked activity in the i.o. branch of NIII (*A*) in an unoperated control fish and (*B*) in a fish in which behavioural observations had suggested that junctions from NIII on the s.o. muscle were repressed. In both control and experimental fish reflexly evoked activity increased in the head down position and decreased in the head up position. These findings indicate that the central connexions of the surgically crossed nerve are not altered.

Explanations of apparent suppression of foreign nerve function

How can the apparent repression of foreign nerve function sometimes observed in behavioural experiments be reconciled with the physiological results which clearly show that functional dual innervation can persist for months?

Myotypic respecification? Reflexly evoked impulse activity was recorded

from the i.o. branch of NIII in intact unoperated control fish and in fish which had undergone nerve crossing. Behavioural observations (see Text-fig. 5) indicated that NIII had innervated the s.o. muscle in all three experimental fish, but then had apparently ceased to function. Text-fig. 8 shows sample records from a control (*A*) and an experimental (*B*) fish. In both, some impulses were recorded when the fish were held level. The activity markedly increased when the fish were tipped 20° head down, and decreased when they were tipped 20° head up. Thus, the reflex output into the surgically crossed nerve (NIII) was essentially similar to that into NIII in the normal fish. This result was obtained from all three experimental animals, and indicates that the central connexions of NIII are not altered when it innervates the s.o. muscle. The behavioural observations therefore cannot be explained by 'myotypic respecification' (Weiss, 1936; Sperry, 1941).

Regeneration of the i.o. muscle? In these experiments, as in those of other workers (Marotte & Mark, 1970*a*; Mark *et al.* 1972) the i.o. muscle was removed to enhance foreign innervation of the s.o. muscle and to facilitate detection of cross-innervation of the s.o. muscle in behavioural tests. Were the i.o. muscle to regenerate and become reinnervated by NIII, its activation during head down tilts would tend to rotate the eye upward in synergy with unoperated rectus extraocular muscles (Text-fig. 2, open circles and triangles, lower left quadrant). The combined action would oppose the downward rotation produced simultaneously when the cross-innervated s.o. muscle is driven by NIII and could mask signs of cross-innervation of the s.o. muscle. This could give the appearance of the loss of foreign innervation on the s.o. muscle.

In the experiments reported here regenerated i.o. muscle fibres were removed in a second operation 14–28 days after the initial surgery. At the time of the final experiment any muscle or connective tissue located in the area normally occupied by the i.o. muscle was attached to the transducer as if it were muscle. Although in some fish it appeared that only connective tissue was present, in all animals which exhibited a behaviour consistent with the loss of foreign innervation stimulation of NIII revealed that some i.o. muscle fibres had regenerated (Pl. 1 *D*) and produced measurable tension (0·03–0·29 g). These tension values are only approximate, for simultaneous contraction of the cross-innervated s.o. muscle may have contributed somewhat to the measured tension. However, in each case, functional i.o. muscle regeneration was confirmed by direct stimulation, and by visual observation of i.o. muscle contraction.

Several preliminary experiments indicate that, when reflexly activated in head down positions, the regenerated i.o. muscle (driven by its own nerve, NIII) can actually rotate the eye upward enough to mask the downward movements produced

simultaneously by the s.o. muscle cross-innervated by the same nerve, thereby causing the apparent repression of foreign nerve function on the s.o. muscle. In four fish the i.o. muscle was removed and allowed to regenerate. In these fish NIII innervated only the i.o. muscle and made no contribution to the s.o. muscle, so the upward eye rotation produced by the i.o. muscle could be observed directly. The tension generated by the i.o. muscle was measured when the muscle had regenerated enough to cause 10–15° improvement in ocular rotation in head down positions, approximately the amount by which eye rotation changed during the apparent repression (see Text-fig. 5). In these fish the regenerated i.o. muscle produced roughly the same amount of tension (0·03–0·39 g) as that produced by regenerated i.o. muscles in those fish in which repression of foreign innervation on the s.o. muscle was indicated in behavioural tests. It seems, then, that a regenerated i.o. muscle can move the eye sufficiently upward to account for the apparent repression of foreign innervation.

Additional evidence was obtained by removing the regenerated i.o. muscle from two fish in which behavioural observations suggested that NIII had initially cross-innervated the s.o. muscle, but that its endings on the muscle had subsequently been repressed. In these fish i.o. muscle removal also removed the behavioural signs of repression. That is, following i.o. muscle removal there was a reappearance of reversed eye rotation in head down positions, indicating that the foreign innervation on the s.o. muscle was still functionally intact.

It seems reasonable to conclude therefore that the regenerated i.o. muscle contracting in synergy with some of the rectus muscles produces sufficient upward eye rotation to obscure behavioural signs of cross-innervation of the s.o. muscle by NIII.

Selectivity of reinnervation?

Evidence from tension measurements. The data presented above indicate that the s.o. muscle fibres can accept and maintain a dual innervation, with the appropriate nerve (NIV) being apparently unable to displace or render functionless established foreign junctions. The question remained, however, whether during simultaneous regeneration of NIII and NIV with both oblique muscles available as targets, there would be selective reinnervation with each nerve exclusively reinnervating its correct muscle.

As a first approach to the problem NIII and NIV were cut near their own muscles and allowed to regenerate in ten fish. The surgery was such as to facilitate the regeneration of the two nerves along their original pathways, and not surprisingly *in vitro* nerve stimulation indicated that each nerve reinnervated its own muscle exclusively. In order to give each nerve an approximately equal chance to innervate both muscles, NIII and NIV were cut near their respective muscles and sutured together in twenty-four fish (Dual-Sim group, see Methods).

Under these conditions there was no obvious preference of a nerve for its original muscle; twenty-three s.o. muscles received dual innervation. In these fish as well as in those in which NIII was crossed to the s.o. muscle before NIV regeneration (Dual-Stag) the average tetanic tension

evoked by stimulation of NIII was not significantly different (Wilcoxon test) from that evoked by stimulation of NIV (Text-fig. 7); the over-all average tension elicited by stimulation of NIII and NIV in all s.o. muscles tested quantitatively was $0{\cdot}52 \pm 0{\cdot}47$ and $0{\cdot}61 \pm 9{\cdot}58$ g (mean $\pm$ S.D., $n = 58$), respectively. On the average functional foreign innervation, whether established prior to or simultaneously with regeneration of the original nerve, was associated with a significant ($P < 0{\cdot}01$) reduction in the tension evoked by stimulation of the appropriate nerve (Text-fig. 7, compare Cont and Rein with Dual-Stag and Dual-Sim).

Investigation of individual neuromuscular junctions. The experiments described above did not exclude the possibility that differences in the effectiveness of appropriate and foreign innervation might occur at the level of individual neuromuscular junctions; that is, the transmitter output of the foreign junctions might be less than that of the appropriate ones. To test this possibility junctional activity was reduced with a modified Ringer solution containing 1·5–6·5 mM-$MgCl_2$, and the mean quantal content of e.j.p.s (m) (del Castillo & Katz, 1954) was measured in twelve unoperated control fibres and in thirty-three experimental fibres.

Spontaneous miniature excitatory junctional potentials (m.e.j.p.s) arising from several discrete junctions were always recorded at a single electrode site, and the smaller, slower m.e.j.p.s from distant junctions tended to skew the distribution of m.e.j.p. amplitudes away from the normal (cf. Burke, 1957). It was not possible, therefore, to estimate the value of m at a single junction by the mean e.j.p. amplitude/mean m.e.j.p. amplitude (del Castillo & Katz, 1954). However, evoked e.j.p.s arising at different junctions on one fibre could be readily distinguished by differences in their latency and time course. Thus, by measuring the amplitude of evoked e.j.p.s of a constant latency in a series of trials it was possible to determine the value of m for e.j.p.s at one junction by calculating $m = 1$ (coefficient of variation of e.j.p. amplitude)^2 and $m = \ln$ (number of trials/number of failures) (del Castillo & Katz, 1954). These methods, which depend only on the number and amplitude of evoked e.j.p.s, give an acceptable estimate of m at one ending on a multiply innervated fibre (Landmesser, 1971; Yip & Dennis, 1976).

With these techniques foreign junctions were indistinguishable from control and regenerated appropriate ones. For example, in fibres examined in 2·0–2·5 mM $MgCl_2$ Ringer the average quantal content (m) and amplitude (v) of e.j.p.s from foreign NIII ($m = 7{\cdot}4 \pm 11{\cdot}1$, $v = 2{\cdot}1 \pm 2{\cdot}2$, mean $\pm$ S.D.; $n = 16$) and from regenerated NIV ($m = 6{\cdot}0 \pm 11{\cdot}0$, $v = 0{\cdot}8 \pm 0{\cdot}7$; $n = 9$) were not significantly different (Wilcoxon test) from those of unoperated control NIV ($m = 3{\cdot}6 \pm 3{\cdot}2$, $v = 1{\cdot}0 \pm 0{\cdot}4$; $n = 6$). In addition, there was no indication that on individual dually-innervated fibres the effectiveness of the appropriate nerve was greater than that of the foreign; in these fibres the average quantal content of e.j.p.s from NIII ($7{\cdot}0 \pm 11{\cdot}1$, mean $\pm$ S.D.; $n = 17$) and from NIV ($4{\cdot}6 \pm 8{\cdot}6$; $n = 17$) were not significantly different.

DISCUSSION

The apparent discrepancy between behavioural and physiological results

In this and in previous reports (Marotte & Mark, 1970*a*; Mark *et al.* 1972; see also Sperry & Arora, 1965) behavioural observations suggested that in some operated fish the endings of a foreign nerve (NIII) which had established functional contact with the superior oblique (s.o.) muscle were 'suppressed' when the embryologically correct nerve (NIV) regenerated to that muscle. However, the physiological experiments described here showed that the function of the foreign innervation persisted unchanged for months after NIV reinnervated the s.o. muscle.

The present results show that the apparent discrepancy between behavioural and physiological results cannot be attributed to 'myotypic respecification' (Weiss, 1936; Sperry, 1941) of the central connexions of the foreign axons (Text-fig. 8). In addition, the suggestion that the behavioural results depend largely upon the responses of slow fibres (see also Sterling & Gestrin, 1975) while the physiological techniques sample only the fast ones (Mark & Marotte, 1976) must also be discounted. Dual innervation of the s.o. muscle was indicated in some behavioural tests (Text-fig. 3), and functional dual innervation was shown to occur unequivocally on both types of fibres.

Rather, the findings reported here indicate that the discrepancy can be attributed to eye movements produced by the regenerated inferior oblique (i.o.) muscle which mask eye rotation produced by cross-innervated s.o. muscles (see also Sperry, 1950). In the present study, i.o. muscle regeneration was shown by physiological tests to have occurred in all of the fish which exhibited a behaviour consistent with apparent repression of foreign innervation on the s.o. muscle. It is interesting to note that in previous experiments (Marotte & Mark, 1970*a*; Mark *et al.* 1972) some i.o. muscle regeneration was described in several fish, one of which also exhibited behaviour consistent with repression of foreign innervation; in the absence of physiological investigations the possibility of o.i. muscle regeneration in other fish cannot be excluded. It seems reasonable to conclude, therefore, that i.o. muscle regeneration could explain behaviour which had been attributed to repression of foreign innervation on the goldfish s.o. muscle.

Possible influence of multiple innervation

The suggestion that polyneuronal, multiterminal innervation of single muscle fibres enables the fibres to reject inappropriate connexions and to retain only the appropriate ones, a capacity that is supposedly lacking in

focally innervated fibres (Mark, 1974) was not supported in this and other studies.

Persistence of dual innervation. In the present experiments there was no evidence either from tension measurements or from intracellular or extracellular recordings that the regenerating appropriate nerve (NIV) displaced foreign (NIII) endings on the same fibre. These results are entirely consistent with data obtained in experiments on focally innervated rat soleus muscle (Frank, Jansen, Lømo & Westgaard, 1975) and multiply innervated fish gill muscle (Frank & Jansen, 1976) in which it was shown in individual animals that foreign innervation persisted unchanged after the original nerve regenerated.

In contrast, displacement of foreign innervation may occur in other species with multiply innervated muscle fibres. In larval *Xenopus* foreign nerve function on the s.o. muscle has been reported to decrease in association with degeneration of displaced axons following regeneration of the appropriate nerve (Fangboner & Vanable, 1974), and in frogs slow axons can regain control of slow muscle fibres which have been previously innervated by fast axons (Schmidt & Stefani, 1976). Suppression of foreign innervation has also been described in urodeles, both on salamander (Mark, 1975) and newt (Yip & Dennis, 1976) muscle. In the latter quantal content at foreign junctions decreases after the original nerve regenerates (Yip & Dennis, 1976), and foreign junctions may ultimately disappear (M. J. Dennis, personal communication). In all these studies indicating a loss of foreign innervation no evidence has been reported of morphologically intact, but functionless junctions, as was suggested for goldfish (Marotte & Mark, 1970*b*; Mark *et al.* 1972).

Selective reinnervation? Earlier behavioural studies in lower vertebrates indicated that when the original nerve to a given muscle was replaced by a foreign nerve, the latter could make functional connexions, consequently producing abnormal movements (Sperry & Deupree, 1956; Sperry & Arora, 1965; Mark, 1965; but see Sperry, 1950). Thus, the finding that in goldfish a foreign nerve (NIII) readily innervated the s.o. muscle when surgically crossed to it (Dual-Stag group) was expected. On the other hand, the same behavioural studies suggested that in these animals *mixed* motor nerves regenerating after simple transection tended to reinnervate selectively their original muscles (Sperry, 1950; Sperry & Deupree, 1956; Sperry & Arora, 1965; Mark, 1965), as did an appropriate nerve implanted into its muscle simultaneously with a foreign nerve (Sperry & Arora, 1965). Thus, the results of experiments reported here in which NIV and the i.o. branch of NIII were sutured together were somewhat surprising, for they did not reveal any selectivity of reinnervation, as judged both from tension studies and from measurements of mean quantal

contents and the frequency of occurrence of fibres with multi-component e.j.p.s.

However, the combined behavioural and physiological studies reported here indicate that earlier behavioural studies demonstrating selective reinnervation may need to be re-evaluated. In the present experiments the observed behaviour correctly indicated the sources of innervation in only one-third of the animals; a co-existing and often extensive foreign innervation was frequently not detected. Contractions of appropriately innervated fibres often obscured eye movements produced by incorrect neuromuscular combinations, giving the false appearance of selective reinnervation (see Sperry, 1950; Czéh & Szekely, 1971). In the absence of associated physiological experiments therefore, behavioural studies, at least on the goldfish, cannot be considered valid approaches to the problem of selective reinnervation.

In contrast to the present findings on goldfish, a more selective reinnervation has been reported to occur in muscles of the salamander (Grimm, 1971; Cass & Mark, 1975) and in mammalian fast twitch muscles (Hoh, 1975). Mammalian slow twitch fibres, however, are reinnervated non-selectively (Hoh, 1975).

From the results of the present investigations and from others in the literature it can be concluded that in fish and mammals functional innervation of a muscle by nerves from two discrete sources can become permanently established; in this experimental situation there is no indication of selective reinnervation. There is evidence that in some species foreign nerve endings may be displaced, and that muscles may be reinnervated selectively. It is clear, however, that neither the species of animal investigated, nor the number of junctions on individual muscle fibres, can be presently related to the maintenance or displacement of an innervation, or to the selectivity or non-selectivity of reinnervation.

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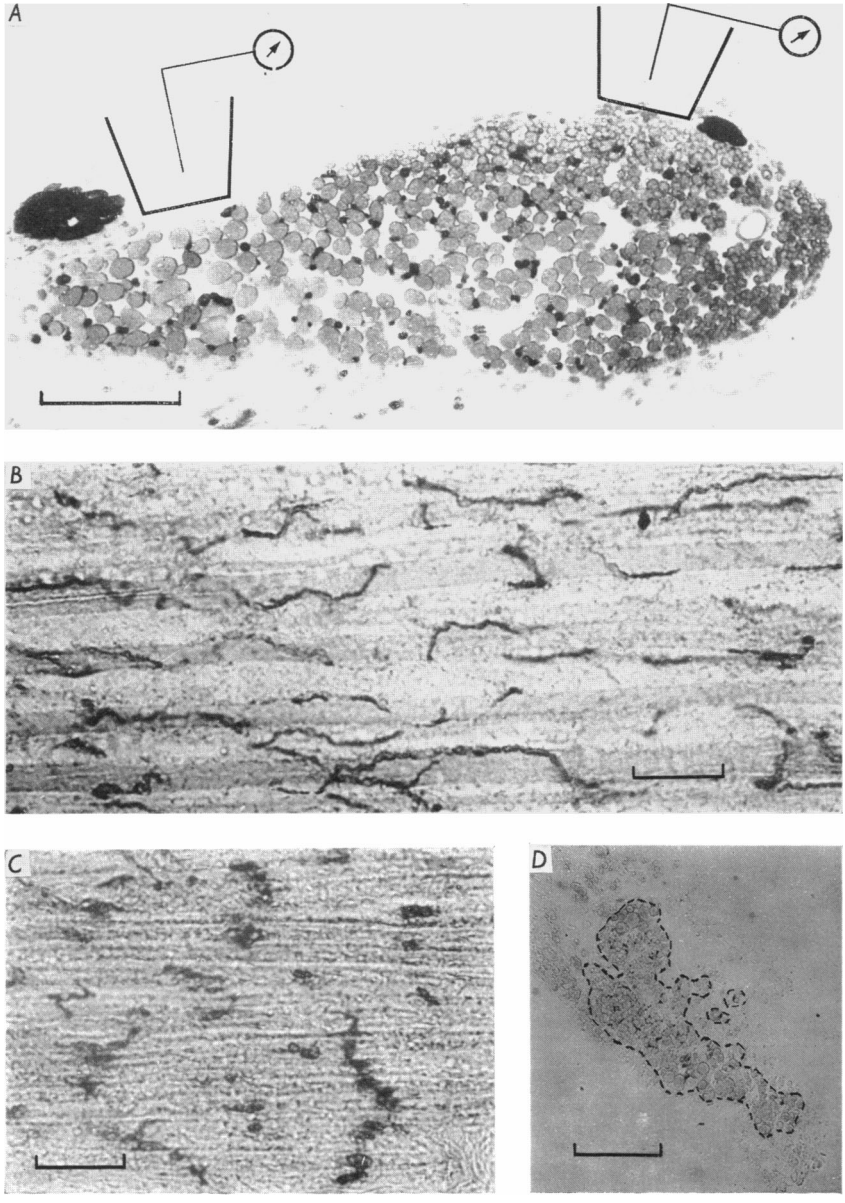
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EXPLANATION OF PLATE

A, cross-section of an unoperated s.o. muscle showing the location of suction electrodes (roughly to scale) placed over several large fast fibres (left) and over small slow fibres (right) on the orbital (outer) surface of the muscle. For intracellular recordings from fast fibres the s.o. muscle was pinned out with its global (inner) surface uppermost. *B* and *C*, acetylcholinesterase-stained fibres on the global (*B*) and orbital (*C*) surface of an unoperated s.o. muscle. Note the difference between the morphology of end-plates on fast (*B*) and slow (*C*) fibres. *D*, a regenerated i.o. muscle which has been stained for acetylcholinesterase; neuromuscular junctions are present. Dashed lines outline the bulk of this muscle. Scale represents 100 μm in *A* and *D* and 50 μm in *B* and *C*.