THE ROLES OF MOTONEURONS AND THEIR MUSCLE TARGETS
IN SYNAPTogenesis DURING REGENERATION
OF A FOREIGN TRANSPLANT

by

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A thesis submitted in conformity with the requirements
for the degree of Masters of Science
Graduate Department of Zoology, University of Toronto

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0-612-40885-X
THE ROLES OF MOTONEURONS AND THEIR MUSCLE TARGETS IN SYNAPTOGENESIS DURING REGENERATION OF A FOREIGN TRANSPLANT

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Rosalind Coulthard, 1998

ABSTRACT

In the crayfish Procambarus clarkii a mixed phasic and tonic nerve from the third root of the sixth abdominal ganglion was allotransplanted onto a denervated, tonic superficial flexor muscle (SFM) of the third abdominal segment. The fastidiousness of the muscle in accepting innervation along with the roles played by the neuron and its muscle target in mandating the regenerating synaptic phenotypes could be assessed by providing such a transplanted nerve with a substantial number of motoneurons and varying synaptic properties. The success of regeneration in forming viable synapses was substantiated through electrophysiology. The excitatory postsynaptic potentials evoked by the stimulation of regenerated neurons included both properties characteristic of the tonic native innervation as well as ones that were novel and more reminiscent of phasic innervation. Thin serial section electron microscopy established the phenotypes of the regenerated synapses in terms of synaptic area, the number of dense bars per synapse and the dense bar length. Some of the regenerated synapses resembled those found in native tonic innervation, while others were clearly novel and more reminiscent of phasic innervation. The presence of these different synaptic phenotypes is indicative of a motoneuronal autonomous programme responsible for dictating structural and functional properties. The muscle shows no favouritism in its acceptance of foreign motoneurons and while it appears to exert minimal influence on the resulting phenotype of the regenerated synapses, it does seem to restrict innervation to lower than normal, never accepting more than three motoneurons.
ACKNOWLEDGEMENTS

I would like to thank my supervisor, C.K. Govind, for his invaluable help and guidance, inexhaustible supply of energy and wonderful sense of humour. Joanne Pearce for her infectious enthusiasm, thoughtful suggestions and ever friendly presence. Kristin Krause for performing the surgeries as only she could do. Raymond Or for his technical support. Lastly, I would like to thank my family and friends, in particular my mom who would have been so proud as well as my dad and D.T., who are proud.
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SUMMARY

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INTRODUCTION

I. Rationale for the study

In the past regeneration studies have been used to better the understanding of synaptogenesis and specificity of neuromuscular interactions (Aguayo et al., 1991; Govind et al., 1973). Synaptogenesis is a complex process requiring both genetic and environmental factors in order for neurons to successfully recognize and form synapses with appropriate targets (Goodman and Shatz, 1993). With the use of genetically altered Drosophila mutants two steps in the process of synapse formation have been defined. First of all, the assembly of active zones within the motoneuron is completed independently of the target muscle and secondly, the presence of differentiated muscle is required to localize active zones at the point of neuromuscular contact (Prokop et al., 1996). However, the ability of foreign nerves to establish viable connections in a number of preparations (Anderson, 1985; Cash et al., 1992; Chiba et al., 1993; Frank and Jansen, 1976) suggests that properties common to all muscle fibres and nerves must be responsible for synaptogenesis.

Whether determination of functional synaptic properties is a result of an autonomous program of the motoneuron or the aftermath of a retrograde influence is currently a highly contentious issue. Many studies in invertebrates lean to the presence of a retrograde factor (Davis and Murphey, 1994) especially those showing that synapses of a single neuron differ in their properties depending on their target tissue. Examples of such studies involve a single neuron contacting two separate targets with differing synaptic properties in crickets and lobsters. In crickets, mechanosensory neurons form depressing synapses on one interneuron and facilitating synapses on another (Davis and Murphey, 1993). Likewise, both facilitating and depressing synapses are found to be made by a single neuron in the
lobster supplying innervation to differing stomach muscles (Katz et al., 1993). A single motoneuron in the crayfish opener muscle has been found to supply both low and high output synapses to different regions of the same muscle target (Atwood, 1976). High output connections with large initial junctional potentials and little facilitation are made on the proximal muscle fibres while low output connections with small initial junctional potentials and considerable facilitation are found on the distal fibres (Atwood and Bittner, 1971).

The robust regenerative powers of the crayfish *Procambarus clarkii* in general, along with the numerous well documented studies performed on the abdominal superficial flexor muscle (SFM) system in particular, make this system an ideal platform for regenerative studies. When the SFM nerve is carefully severed so the proximal stump is still in contact with the muscle, reestablishment of neuromuscular connectivity maps takes a mere 8 weeks (Ely and Vélez, 1982). Further manipulations of SFM nerve to its target included the rerouting of the nerve to innervated targets (Hunt and Vélez, 1989b), to reduced targets (Clement et al., 1983; Hunt and Vélez, 1989a), on ectopic sites of the muscle surface contrary to the accustomed growth route (Goransson et al., 1987; Hunt and Vélez, 1989a), and to targets located in different segments altogether (Worden et al., 1987). All these manipulations gave testimony to the ferocity of regeneration pursued in this system, for in all cases regeneration of neuromuscular maps were essentially restored. Further experiments established the ability to successfully allotransplant an SFM nerve with associated ganglia from one animal to another (Krause and Vélez, 1995). The epitomy of the regenerative powers in this system was perhaps best exemplified with the successful allotransplantation of the deep third root which normally innervates a phasic muscle system to the tonic SFM (Krause et al., 1998). This success opened the door for further regeneration studies in an adult peripheral system.
The ability to successfully allotransplant foreign nerves provides the opportunity to set up a paradigm where motoneurons, both tonic and phasic, can be transplanted and made available for regeneration. In the present study this was accomplished by transplanting the third root of the sixth abdominal ganglion which normally supplies innervation to the uropod muscles to a denervated SFM. This foreign nerve root contains at least 19 motor axons, both phasic and tonic in nature (Higuchi, 1992) and by making such a choice available to the tonic SFM, one can better assess the roles played by the motoneuron or its target in the ensuing connections that are made. Through serial thin-section electron microscopy the fine structure of these foreign regenerated synaptic connections in conjunction with their electrophysiological properties can be assayed. Consequently, the respective influences of motoneuron and muscle upon one another in the synthesizing of these regenerated synaptic connections can be examined. Thus my study will examine the respective roles of the motoneuron and target muscle in determining synaptic phenotypes in an established adult system. If the regenerated synapses are uniform in nature resembling those seen in the native innervation, this will suggest the muscle plays a role in limiting the type of connections that are made. However, if the opposite is true and the regenerated synapses vary from their native counterparts, this suggests the motoneuron is more instrumental in mandating the type of synaptic connections made. Embryological studies in Drosophila point to the motoneuron being more responsible for its resulting synaptic phenotype, based on ultrastructural analysis (Prokop et al., 1996). On the other hand, amongst other invertebrates, single neurons which form synapses with differing functional properties point to the existence of a retrograde signal (Atwood and Bittner, 1971; Frank, 1973; Atwood and Wojtowicz, 1986; Davis and Murphey, 1993, 1994). To resolve these opposite views, I have undertaken to examine regeneration of synaptic connections in the SFM by a foreign nerve.
II. Background Information

A. The SFM preparation

The superficial flexor muscle (SFM) in *Procambarus clarkii* has proven to be a very advantageous muscle in neurophysiological studies for a number of reasons. As with all arthropods, the number of neural elements involved is greatly restricted in comparison to higher animals. Consequently one can study individually identifiable axons on a single cell level. Due to its anatomical make-up, the SFM preparation in particular provides relatively easy accessibility and visualization. This, in conjunction with the extent to which its physiological properties have been studied, has further made it a desirable choice in examining the effects of an array of surgical manipulations upon its neural circuitry.

i) Anatomy

The SFM is a thin sheet of muscle lying on the ventral side of the crayfish abdomen, and is responsible for its delicate flexor control (Kennedy and Takeda, 1965b). Anatomically it consists of two layers of muscle, distinguished by their differing attachments to the sternum (Fig. 1). Each layer consists of a single layer of cells although at times an occasional third muscle fibre situated between the two is seen. The ventral-most layer runs the whole width of the muscle while the dorsal merely extends over the lateral half; both layers comprised of fibres running parallel to one another. All in all, the total number of fibres constituting both layers equals $42 \pm 3$ with approximately 23 fibres being visible from the dorsal surface and which could individually be mapped for their innervation (Vélez and Wyman, 1978a).

The nerve supplying the SFM emerges from the central nervous system as a separate superficial branch of the third root. The muscle receives complex, polyneural innervation from 6 serially homologous axons; 5 of which are excitatory and the last inhibitory
Figure 1. Superficial flexor muscle in an abdominal segment of crayfish is normally innervated by tonic motoneurons whose axons travel in a thin branch of the third root of the ganglion. Cutting this nerve denervates the muscle and allows the allotransplant to regenerate and form new innervation. The third root of the sixth abdominal ganglion was allotransplanted underneath the denervated muscle. The transplanted nerve regenerates onto the muscle dorsal surface and it is stimulated to test for reinnervation while recording intracellularly from muscle fibres.
(Kennedy and Takeda, 1965b). They are named in concurrence to their individual spike heights, axon 1 being the smallest, 6 the largest and 5 acting as the inhibitor. Each of the 5 excitatory axons is distinguishable by the individual amplitude and facilitation properties its excitatory postsynaptic potentials (EPSPs) display (Kennedy and Takeda, 1965b). The somata of these 6 motoneurons are located in the abdominal ganglia and by virtue of their placement in the ganglia fall into 3 groups; anterior, medial, and posterior. The anterior group deviates most from serial homology and includes axon 1, the most unique of the axons in being the only one with its soma placement in the next caudal ganglion (Wine et al., 1974). The medial group includes axons 3 and 5. The excitor, axon 3, has its somata lying more centrally with neurites ascending up the midline as compared to the inhibitor, axon 5, which has a more lateral placement. The posterior group which includes axons 2, 4 and 6 and whose somata lie in close proximity to one another. Axon 1, due to its posterior placement, lacks the presence of any somata in the last abdominal ganglion (Mittenthal and Wine, 1978). Due to the disparate placement of each axon’s cell body from one another, the ability of independent identification and manipulation is achieved.

The likely neurotransmitter used by the excitors is L-glutamate (Evoy and Beránek, 1972; Hildebrand et al., 1974), a depolarizing agent used extensively throughout the arthropod kingdom. However, there is a possibility that the largest of the excitatory axons (axon 6) may release the more popular neurotransmitter choice of vertebrate motor neurons, acetylcholine (Futamachi, 1972). The best candidate for the neurotransmitter used by the common inhibitory neuron (axon 5) is gamma-aminobutyric acid (GABA) (Otsuka et al., 1966). More recently co-transmitters, in particular the pentapeptide proctolin, have also been found via immunohistochemistry and high-performance liquid chromatography to be released from 3 excitatory axons viz. axons 1, 3 and 4 (Bishop et al., 1984).
ii) **Innervation**

The axons are considered to be tonic in nature, most exhibiting some degree of spontaneous activity and thus supplying the muscle with a constant barrage of neural input. Some of the motoneurons to these flexors may further act as endogenous oscillators (Tatton and Sokolove, 1975). Accordingly, the muscle is deemed to be in the realm of a "slow" type muscle, substantiated by its relatively longer sarcomere length (9 - 11 μm) (Vélez and Wyman, 1978b) characteristic of crustacean "slow" muscle (Atwood, 1976). The ability to evoke all-or-none responses is rare; graded tension is achieved through summation and or facilitation of excitatory postsynaptic potentials (EPSPs) (Evoy et al., 1967). However, neither the motoneurons nor the muscle are uniform; they exhibit a wide range of properties. The smaller axons, such as axons 1 and 2, exhibit the highest degree of tonic activity giving the largest spontaneous firing frequencies (axon 2 fires at a frequency of around 17 Hz) and the smallest EPSPs. They play a major role in maintaining the flexor tone present in an undisturbed animal. Conversely, axon 6 boasts the largest EPSPs, capable of greater facilitation and tending more to fire in phasic bursts (<1 Hz) (Kennedy and Takeda, 1965b). Consequently it is more suited in effectuating stronger reflexes requiring comparatively faster movement (Evoy et al., 1967).

The motoneurons vary in the amount of innervation they provide to the SFM, ranging anywhere from 40 to 100% of the total muscle fibres (Vélez and Wyman, 1978a). Past studies on the extent of innervation by the inhibitor axon, axon 5, suggested that less than half of the muscle fibres were recipients of its input (Kennedy and Takeda, 1965b). More recent studies involving both pharmacological and electrophysiological methods have proven this conclusion errant, showing that the inhibitor in fact innervates 100% of the muscle fibres (Evoy and Beránek, 1972; Vélez and Wyman, 1978a).
Each SFM muscle fibre is innervated by at least 3 axons, two excitatory and one inhibitory. (Kennedy and Takeda, 1965b; Vélez and Wyman, 1978a). Kennedy and Takeda (1965b) noted that there were random broad regional differences in the distribution of the motor axons and their innervation combinations. However, Vélez and Wyman (1978a) found that although innervation appears random when muscle fibres are compared individually, population studies show the presence of some clear trends. They attempted to prove that not only the location, but also the strength of synapses in the SFM were probabilistically controlled and concluded that a medial to lateral gradient existed across the muscle based on the probability of a given axon innervating a said fibre. This gradient exacted minimal or maximal innervation by nerves at the borders of the muscle with innervation proceeding across the muscle in a nearly linear function. It was proposed that each axon reads this gradient using a different proportionality constant resulting in their different innervation patterns. The two smaller axons (axons 1 and 2) exhibited increased innervation in a lateral to medial direction while axons 4 and 6 went from medial to lateral. Axon 3 essentially acted as a common excitor innervating all the muscle fibres. Since this gradient is thought to determine the mean rate of the random process of innervation, it also directly controls mean synaptic strength as measured by average EPSPs. Trends in synaptic strength parallel those seen in innervation probabilities where axons 1 and 2 give greater average EPSPs in a lateral to medial direction. Conversely axons 4 and 6 show increases in a medial to lateral fashion, while once again axon 3 essentially evokes uniform EPSPs across the entire muscle. Thus, Vélez and Wyman (1978a) have hypothesised that the SFM has a single gradient across its medio-lateral axis which determines both the probability and strength of innervation.
iii) Muscle properties

Tension development exists tonically in the SFM, thus a single motoneural impulse is unsuccessful in achieving tension in the muscle. Correspondingly, the muscle fibres exhibit long latency periods and a large frequency to tension ratio (Evoy et al., 1967). Although as a whole the muscle is considered to be of the "slow" variety, there is still great variance in the properties amongst its individual fibres. Input resistance, time constants, resting potentials and the size of the muscle fibres themselves are all properties found to vary across the muscle. Evidently input resistance and time constants are related to muscle fibre size. The lateral third of the muscle contains bigger fibres with lower input resistance, shorter time constants and greater resting potentials. It demonstrates the most consistency in these values. On the other hand, the most medial third shows the greatest variance although the majority of fibres possess greater values of input resistance, longer time constants and lower resting potentials. Correspondingly, they tend to be smaller in size. As would be expected, the central fibres are intermediate in their properties (Vélez and Wyman, 1978b).

The degree of tension effectuated by axons 1, 3 and 4 is enhanced by the concomitant release of proctolin (Bishop et al., 1987). Proctolin was found to be dependent on the depolarising EPSPs produced by the conventional neurotransmitter (most likely L-glutamate) for by itself it is unable to initiate muscle tension, much less affect resting membrane potentials. Thus, in order for proctolin to potentiate muscle tension it must somehow positively affect the excitation-contraction coupling mechanism although the exact cellular mechanisms involved are still unknown (Bishop et al., 1987). Obviously an advantage of using proctolin is efficiency related, resulting in the requirement for less neural activity. Interestingly, it was further found that of the minimum two excitors a muscle fibre receives, one of these is proctolinergic while the other is not (Bishop et al., 1987).
B. Degeneration and Regeneration in Crayfish Peripheral Nerves

Crayfish (*Procambarus clarkii*) have been established as very beneficial models in neurophysiological studies. In particular their relatively few neural components allowing for experimentation at a single cell level and comparatively large muscle cells accommodating easy accessibility for electrophysiological and anatomical examination make them prime candidates. This in corollary with the finding that upon decentralization their nerves exhibit extraordinary staying powers (Hoy et al., 1967; Hoy 1969; Nordlander and Singer, 1972; Atwood et al., 1974; Kennedy and Bittner, 1974; Vélez et al., 1981) has further served to increase their usefulness in regeneration and developmental studies.

i) Resistance to Degeneration Following Axotomy

The amazing capacity to maintain both morphological and functional synaptic junctions in peripheral nerves is somewhat unique to the crayfish. Within mere hours after axotomy in vertebrates the onset of both structural and functional degeneration takes place (Cajal, 1928; Young, 1942). Proximal to the cut, retrograde degeneration of the cell body is observed and distally Wallerian degeneration of the axon. Within only one to two days, complete junctional silence is established (Titeca, 1935; Hunt and Nelson, 1965).

Amongst some arthropods, such rapidity in degeneration is also seen. Decentralized nerves in the walking leg of the cockroach lose their capability to propagate a spike in 3 to 5 days. At this time, stimulation of EPSPs is impossible and all spontaneous activity has ceased. This is accompanied by a 30% decrease in resting membrane potentials of the muscle (Jacklet and Cohen, 1967). Degeneration in neuromuscular junctions of the walking legs in the locust furnish intermediate findings (Usherwood, 1963b). In the first week after
transection, junctional properties remain similar to those seen in controls. Subsequently EPSPs with distorted waveforms and progressively smaller amplitudes are observed. At approximately 3 weeks the ability to evoke EPSPs desists altogether. Spontaneous activity also becomes erratic past the first week, occurring in bursts, then completely halting around the 32nd day after axotomy.

Only a couple of examples have been discovered exhibiting a retardation in the onset of degeneration. Sectioned distal motor axons of octopus have been observed to persist for up to 34 days (Wilson, 1960). In the oesophageal connectives of locusts and mantids only 2% of neuronal fibres were found to degenerate although more than half were “soma-less” (Boulton, 1969). It is thought that these differences in the maintenance of excitability upon decentralization may be a result of an organism’s ability to control resting potentials (Hoy, 1969). For example, resting potentials in the walking leg of the locust decrease by 10-30% after the first week of denervation and by 60% after 3 weeks. These descending steps in resting potential coincide strongly with junctional ability (Usherwood, 1963a). Resting potentials in the crayfish muscle show no trends of diminution but rather a slight increase in value (Girardier et al., 1962). This may attest for the nerves’ delayed degenerative properties.

ii) Resistance to Degeneration in the Crayfish

As early as 1960, Wiersma noted the slowness of degeneration in crustacean motor axons. One of the first studies examining this remarkable perseverance of severed nerves was done on the crayfish opener muscle found in the claw. Transection of motor axons (consisting of one inhibitory and one excitatory axon in the opener muscle) followed by electrophysiological monitoring uncovered the ability of distal motor segments to remain fully functional for up to a year (Hoy et al., 1967). Assuming that nerve terminals remain
intact, the majority of distal nerves examined in and around the 100th day post-lesion were found to have EPSPs with similar amplitude and waveforms as those seen in intact preparations. Furthermore, the frequency and amplitude of miniature EPSPs and facilitation ratios of EPSPs also remained within normal parameters. For periods exceeding 100 days after decentralization, nerve terminals began showing signs of decreased excitability. When shorter segments of the distal stump were left over the muscle, degeneration was accelerated (Bittner, 1973; Atwood et al., 1974; Vélez et al., 1981). For example, axon remnants of 2-3 cm elicited degeneration in 150 to 300 days while those of only 1-2 mm only took 20 to 50 days. More recently, in a study directly aimed at analyzing synaptic properties and whether or not they live up to their intact counterparts, recorded EPSPs were found to be somewhat lower (Vélez et al., 1981). This depression occurred within 10 days of axotomy, being more prominent in the proximal and distal muscle fibres. By 80 days experimental animals showed a 10-30% decrease in EPSPs compared to controls. Because muscle resistance has been found to remain stable after decentralization (Boone and Bittner, 1974), this reduction in EPSPs is most likely due to retrenchment of neurotransmitter release. Although a change in postsynaptic glutamate sensitivity could be responsible, previous work has downplayed this possibility (Frank, 1974).

Analysis by electron microscopy confirmed these physiological findings (Nordlander and Singer, 1972; Atwood et al., 1974; Kennedy and Bittner, 1974; Vélez et al., 1981). No signs of degeneration were seen 1 cm distal to the point of transection for many months. Axons contained normal appearing endoplasmic reticulum, longitudinally situated microtubules and peripherally orientated mitochondria. Their nerve terminals disclosed the presence of synapses and synaptic vesicles. Muscle morphology also appeared normal, displaying regularly structured contractile filaments and tubules. The axonal area exposed
as a result of transection was soon sealed over by an intact membrane. This area consisting of about 0.1-0.2 mm adjacent to the lesion underwent a reaction similar to that seen in vertebrates. Within 48 hours the glial covering, particularly in the innermost adaxonal layer, appeared swollen. This swelling was accompanied by an increase in rough endoplasmic reticulum, mitochondria, Golgi apparatus and vesicles; a condition which continued to persist for many months (Atwood et al., 1974). Likewise, both the disconnected ends became swollen and were found to contain mitochondria, multivesicular bodies, dense bodies, large clear vacuoles, aggregating small vesicles, tubular and fibrous elements in the periphery as well as sparsely distributed glycogen-like granules. Any debris located between the two endings was soon disposed of by assembled phagocytes.

When degeneration in the nerve terminals finally took place after a staggering 368 days in one case (Kennedy and Bittner, 1974), it was indicated by the presence of irregular mitochondria, vacuoles and dense granular sarcoplasm. In addition, there was a decrease in the number of synaptic vesicles with those remaining tending to clump together. The observed dense granular sarcoplasmic reticulum also exhibited enlarged clefts pointing to possible retraction and degeneration of nerve terminals. Muscle structure was seen to disintegrate. A predominance of mitochondria degenerated leaving behind dark-staining, debris-filled vacuoles and contractile filaments became obscured, Z-lines desisting altogether. Lastly, a hypertrophy of the glial and connective tissue on the surface of the muscle was also apparent.

Incidental work done on both the deep (DFM) and superficial (SFM) abdominal flexors in the crayfish provided comparable results to those of the opener muscle system (Hoy, 1969). The decentralized nerves and muscle fibres in question also remained resistant to degeneration over an extended period, maintaining functional junction transmission with
only slight depression in EPSP amplitude (Bittner, 1977). The SFM system showed the least endurance: spontaneous activity stopped immediately following axotomy and degeneration was detected in just over a month (Hoy et al., 1969; Ely and Vélez, 1982). Prior to this, the neuromuscular junctions remained electrically excitable displaying close to normal properties in terms of waveform, amplitude and facilitation rate. Often not all 6 axons could be evoked into activity, however, there were no axons that showed a greater statistical chance of survival over the others (Ely and Vélez, 1982).

**iii) Trophic Factors**

The occurrences of neural perseverance following axotomy (Nordlander and Singer, 1972; Hoy et al., 1967; Hoy 1969; Atwood et al., 1974; Kennedy and Bittner, 1974; Vélez et al., 1981) bring into question the trophic factors allowing for this long-term survival. Such findings dispute the enduring perception of the cell body as being the trophic centre of the nerve cell. Possible alternatives for biosynthetic support include: a) the capacity of the axon segment to store enough substances to be self-sustaining; b) the axon itself possessing its own synthetic machinery, thus being able to manufacture metabolites to meet its demands; c) nutritional factors being supplied by the surrounding glial cells; and d) other exogenous sources, such as muscle and hemocoelic fluid, supplying nutrients.

The finding that RNA is present in axons, gives credence to the possibility that a neuron may contain its own synthetic apparatus. Although the type and origin of this RNA is still unknown, it has been suggested that it may be supplied by the neuronal sheath cells (Pevzner, 1965; Singer and Green, 1968). The ability of decentralized nerve terminals to maintain EPSPs and demonstrate facilitation are an indication of the nerve's continuing competence in storing and synthesizing neurotransmitter. This bolsters the recycling hypothesis of endocytosis and points to a mechanism for local synthesis (Atwood et al.,
1972). The finding that RNA synthesis in the ventral nerve cord of crayfish is possible without the attachment of a cell body (Anderson et al., 1969) further augments the viability of these axons being able to synthesize their own requirements.

The role of glial cells as potential trophic centres is also quite plausible. The swelling and accumulation of organelles observed in this layer (Hoy 1970; Nordlander and Singer, 1972; Atwood et al., 1974; Kennedy and Bittner, 1974) along with it becoming the predominant cell type following nerve transection (Hoy, 1969), may be indicative of an increase in synthetic activity. The capability of glial cells in vertebrates to transport substances from its surroundings through sheath cells and into axons (Singer and Salpeter, 1966), lends extra support to this theory. In some arthropods (Kuffler and Nicholls, 1964) the ability of ions and large molecules to diffuse from hemocoelic fluid, through clefts situated between neighbouring glial cells straight to the axonal surface, has been documented. While this hemocoelic origin may account for some exogenous trophic support, any part played by the muscle in maintaining the distal segment appears to be nonexistent. Immobilization, and more severely, tenotomy of the muscle was shown to have minimal effects on EPSP amplitude and facilitation evoked in severed distal nerve segments (Atwood et al., 1974).

Interestingly, tenotomy did have a drastic effect on the structure of axotomized muscle (Atwood et al., 1974). In preparations where the muscle was not interfered with, morphological integrity of the muscle fibres is maintained for at least 100 days. However, in decentralized and tenotomized muscle, atrophy as characterised by the obscurement of contractile filament organization and the obliteration of Z-bands, was detected in only 20 to 30 days. Although this corrupting process was accentuated in cases where nerve terminals had degenerated, the otherwise functional terminals did not interfere with atrophying muscles. Therefore, while the muscle is trophically dependent on the normal activity of
motor axons as shown by its maintained structural integrity in the presence of functional terminals and coincident decay when terminals start degenerating, this alone is not sufficient for muscle survival. Passive tension must also play a role along with neurotrophic factors. It has been proposed that a local trophic effect triggered by motoneuron activity in releasing the required nutrients from glial cells may transpire (Bittner, 1973; Atwood et al., 1974; Bittner and Johnson, 1974).

iv) Regeneration in the Crayfish
Regeneration in the SFM was less successful than that encountered in both the opener and deep flexor muscles (DFM). After being lesioned, the DFM was able to attain functional regeneration in the relatively short time span of 20 to 30 days (Hoy et al., 1967). Likewise, the opener muscle could reach full functional levels of regeneration in 20 to 30 days if the nerve had been cut, and only 10 to 20 days if the nerve had been crushed. Through structural analysis of the severed opener nerve, regrowth in the proximal stump was shown to commence within a week (Nordlander and Singer, 1972). The time taken for proximal end outgrowth to reach that of the distal end, averaged around 4 weeks. In the DFM and opener muscle systems, regeneration takes less time than that taken for the onset of degeneration. Their time course of around 30 days, is akin to that of regeneration seen in the cockroach (Guthrie, 1967; Jacklet and Cohen, 1967). However, unlike the crayfish, full functional capacity of junctions in the cockroach is never reestablished (Guthrie, 1967).

The mechanism whereby crayfish achieve regeneration is debatable. It is not disputed that central outgrowth of the proximal segment, as also demonstrated in all vertebrates and some arthropods, is indeed one device used by the crayfish. The contention arises when the possibility of axonal fusion as an alternative method is broached. Nordlander and Singer (1972) disagreed with the contention that fusion was a viable consideration. They cite their
morphological finding of the presence of satellite axons in lesioned opener nerves as direct evidence of centrifugal regrowth. These satellite axons due to their incorporation of orientated microtubules and vesicles, are argued to be sprouts from the proximal nerve stump. They are preceded by the formation of double membrane-bound compartments in the swollen adaxonal glial layer. Four weeks following transection only a few such compartments are seen. By the twelfth week this number is greatly increased with the compartments themselves becoming more axonal in nature. During regeneration, satellite axons are found to take up more room in the axonal sheath and are first seen concentrated near the area of lesion, later extending in progressively larger numbers to more distal regions. Nordlander and Singer (1972) presented this as direct evidence for centrifugal regeneration.

Conversely, based solely on the physiological data they obtained, Hoy and his colleagues (1967) extolled the virtue of the fusion theory, vilifying serial regeneration as the only form of regrowth. The physiological evidence in the opener muscle system included: a) the coincidental return of function for the opener and more distally located stretcher muscle; b) the inability to detect greater numbers of EPSPs even though the distal nerve segment had not degenerated and was still viable; c) the lack of extraneous axons detected via methylene blue staining. Reconciling the two theories, Kennedy and Bittner (1974) concluded that only in those animals exhibiting delayed regeneration (requiring more than 200 days recovery) were there indications of central outgrowth. While agreeing with all of Nordlander and Singer's (1972) findings in regard to the first 2 cm of the lesion, Kennedy and Bittner (1974) never observed these newly sprouting axons reaching the muscle in animals which showed regeneration in less than 100 days. However, animals taking longer than 200 days did exhibit these structures over the entire muscle, a finding corroborated by Atwood et al. (1974). The connections made in this latter case were not fully functional,
probably due to the inability of the animal to reinnervate all of the over 60,000 terminals originally present. Thus in cases where regeneration in the opener muscle system occurs in less than 100 days, outgrowth is observed adjacent to the lesion as the proximal end is sending out shoots to its distal counterpart. Upon fusion of the proximal and distal ends, full function is recouped along with the absence of further sprouts over the muscle. Animals requiring longer than 200 days for regeneration do show signs of central outgrowth with abnormal junctional connections resulting in impairment of muscle use.

The obvious advantages in implementing the fusion theory are twofold. Firstly, due to the lesser degree of synthesis required, a great metabolic advantage is met. Secondly, such a mechanism eliminates the daunting task of correctly reestablishing every peripheral terminal. If the fusion theory holds true, only a single correct reconnection for each axon in the nerve would be required.

C. Neural Transplantation in the Crayfish SFM

i) Transplantation of native nerves
Alluded to earlier, the SFM rarely exhibits regeneration unless the nerve is carefully cut laterally to the border of the medial fibres ensuring attachment of the proximal end to the muscle fibres (Ely and Vélez, 1982). If such precautions are not met and the nerve is cut close to the ventral cord, the resulting space required to be breached by regrowth often proves too daunting a task. In the few cases where this obstacle is overcome (Hoy, 1969), functional regeneration is rarely implemented in all 6 axons. The detection of spontaneous activity in the distal segment, initiated either autonomically or by tactile stimulation, signalled the incidence of regeneration. In cases where less than the full complement of axons underwent successful regeneration, the unsuccessful nerves were still present and
functionally excitable in the distal segment. Even after complete regeneration, not all muscle fibres were reinnervated, since they failed to produce either spontaneous activity or evoked EPSPs. Like the opener and deep flexor muscles, the accomplishment of regrowth is enhanced when nerves are crushed instead of cut. The act of crushing preserves the nerve’s intact sheath keeping the two nerve ends confined and thus expediting the meeting of the outgrowing proximal end to its distal counterpart.

When transection of the SFM nerve is accomplished as explained above, a 55% success rate in regeneration is achieved (Ely and Vélez, 1982). However, unlike the opener and DFM muscle preparations, in the case of the SFM neuronal fusion was not observed. Regeneration was first observed at 3 weeks at which point the transected distal nerve had lost all excitability and thus also the capability of reconnection. In accordance, the distal segment was always visually distinct from its outgrowing proximal component.

Interestingly, consummation of the connectivity maps, both in terms of innervation probabilities and synaptic strength, was observed in regenerating SFM motoneurons (Ely and Vélez, 1983). This gave validity to the proposition that a positional gradient existed over the muscle surface influencing both the probability and strength with which a nerve would make connections (Vélez and Wyman, 1978a). It was postulated that each nerve responded to this gradient with a different proportionality constant resulting in each neuron’s unique pattern. The reinstatement of these patterns after regeneration was completed by all 5 excitatory axons within 10 weeks. Surgical manipulations in these developmental quests are made possible by this relatively fast regeneration period and the SFM’s accessible location immediately adjacent to the cuticle.

It was hoped that through a series of experiments comprised of various surgical
manipulations the factors responsible for the reinstatement of connectivity maps could be established. These SFM manipulations included: a) regeneration studies following nerve transection (Ely and Vélez, 1982); b) the transplantation of the contralateral nerve on both denervated and innervated muscles (Hunt and Vélez, 1982; Hunt and Vélez, 1989b); c) regeneration in muscles where the target area has been decreased either by the removal of lateral or medial fibres (Clement et al., 1983; Hunt and Vélez, 1989a); d) ectopic placement of transplants on the muscle surface with varying target areas (Goransson et al., 1987; Hunt and Vélez, 1989a); e) transplantation to denervated muscles of nerves from differing segments (Worden et al., 1987). Although not fully comprehensive, a vague understanding of possible mechanisms involved for each axon was attained. It appears that at the onset of regeneration these axons are driven by preprogrammed genetic cues, making synaptic connections haphazardly regardless of environmental factors. Such indiscriminate innervation is short-lived lasting no longer than 5 weeks and perhaps imperative in providing the neuron with the opportunity to gain a foothold. This foothold in turn allows for trophic requirements to be met. The next stage progresses to more discrete contacts, either a result of environmental prompts and/or competition between fellow axons. Lastly, periods of hyperinnervation and retraction are often observed, perhaps a consequence of the fine tuning required to make the muscle fully functional. Different responses from individual axons were observed in response to the same environmental deviations as well as differing sensitivities to target removal. However, these varying responses resulted in the ability to ultimately re-establish connectivity maps.

In an attempt to determine the extent to which the SFM axons will reinstate their connectivity maps, a number of further experiments on denervated muscles were performed. As demonstrated by all the excitatory axons, an effect was realized in the presence of an intact nerve (Hunt and Vélez, 1982; Hunt and Vélez, 1989). Other systems
studied thus far have shown that an innervated muscle deters further innervation by other nerves (Jacobsen, 1978). This hindrance could either be due to the repression of growth stimuli or to a mechanical and/or chemical deterrent provided by the intact neuromuscular junctions. The SFM was the first preparation to show some hyperinnervation by the transplanted neuron despite the circumstance of an intact nerve. Although the lateral fibres were resistant to hyperinnervation, the medial fibres were not. The resulting extraneous innervations were far less rambunctious in their pursuits with the presence of an intact nerve. Whether the medial fibres' acceptance of more connections is due to the existence of free synaptic sites or an indication of differing muscle properties is still unknown.

Whatever the cause, this decrease in growth rate demonstrated that the first nondiscriminate stage of reinnervation must also involve some environmental factors, although the preprogrammed stimuli to axotomy still appears to be the greatest impetus.

Successful re-establishment of connectivity maps was still observed when the transplant was made in the centre of the muscle field (Goransson et al., 1988), in a different abdominal segment (Worden et al., 1988), and even in allotransplants placed on the ventral surface of the lateral muscle edge (Krause and Vélez, 1995). In order to retain the somata of the allotransplanted axons, the ganglia containing their somata (the third and fourth abdominal ganglia) were transplanted along with the ventral nerve cord connecting them. The second of these experiments demonstrated that when the normal environmental cues are either absent or weak, as expressed in a differing abdominal segment, regeneration is driven by an inherent preprogramme where the majority of axons reacquired the connectivity maps of their original segment (Worden et al., 1988). Once again the proliferation of synaptogenesis is primarily seen at the site of transplantation ratifying the proposed nondiscriminate first stage.
ii) Allotransplants of Native and Foreign Nerves

Surprisingly, despite their deprivation of normal interactions, allotransplants of SFM nerves from other animals were successful, showing signs of reinnervation within 8 to 10 weeks (Krause and Vélez, 1995). In order for such transplantations to work a substantial stump of the third segmental SFM nerve root along with the third and fourth ganglia and their connecting ventral nerve cord must be incorporated. The host SFM muscle is usually located in the third abdominal segment and hence inclusion of both the third and fourth ganglia is necessary to ensure survival of all the axons that make up the SFM nerve as their cell bodies are contained in both. In comparison to conventional regeneration cases achieved via rerouting, the resulting synaptic connections exhibited smaller EPSPs with no facilitation. Along with the unexpected finding of viable synaptic connections, these allotransplants also demonstrated the dependency of regeneration on positional cues. Despite the placement of the transplant in a position 180 degrees removed from the site of natural regeneration, the majority of axons are still able to resurrect their normal connectivity patterns. Only 60% of the fibres in the SFM were found to be reinnervated suggesting that full innervation is not a prerequisite for the reestablishment of connectivity maps.

Along with the 180° displacement, these transplants were also placed on the ventral side whereas growth of this nerve usually occurs on the dorsal surface. Presumably these transplants perceived this contradictory placement, appearing to grow through the muscle fibres and assuming the bulk of their regrowth on the conventional dorsal side. This supposition was confirmed by electron microscopy where nerve sprouts were observed running between muscle fibres (Krause et al., 1996). Once these sprouts reach the dorsal side, fasciculation of the transplant to the old distal stump is believed to occur, helping in
the guidance of reinnervation.

Morphological studies provided further corroborations (Krause et al., 1996). Analysis of the transplanted ganglia along with the connecting ventral nerve cord, showed regions with intact axon tracts, neuropil and somata. The third ganglion contained 20 healthy somata for as long as 30 weeks after transplantation. Likewise, the fourth ganglion revealed 14 somata. These findings more then attest for the required survival of the five SFM cell bodies (from axons 2 through 6) in the third ganglion and the 1 cell body (from axon 1) in the fourth, for the success of these allotransplants to be valid. Although identification of these somata was impossible they were found to receive excitatory and inhibitory connections, as were dendrites found in the neuropil. Spontaneous activity is probably a consequence of these viable interneurons. The capability of these ganglia to persist was explained by the finding of many blood vessels within them. The presence of blood cells within these vessels suggests that the crayfish is able to infiltrate the transplanted ganglia with blood vessels providing it with its trophic requirements. Such infiltration has previously been recorded in crustaceans (Guchardi and Govind, 1990). Analysis of the thickened regenerating nerve found on the muscle’s dorsal surface showed numerous axon profiles denoting the incidence of sprouting. Synaptic terminals were found to be well defined with clear synaptic contacts and active zones. They were often found lying in close proximity to degenerating nerve terminals suggesting a mechanism by which specificity can be re-established. The number and size of synapses and active zones were not analyzed, so whether disparities in these values exist that result in the depressed EPSPs, is not known. Granular sarcoplasmic reticulum, a highly specialized muscle component where nerve terminals are found, was at times observed without its nerve terminal complement. This lies in agreement with the finding that only 60% of the muscle fibres were reinnervated.
The amazing regenerative powers of the SFM system were further exemplified by the successful allotransplantation of a foreign nerve in lieu of the SFM nerve that normally innervates the muscle. In this case the deep third root which supplies innervation to the deep flexor muscles along with its corresponding ganglion containing the nerve’s cell bodies, was found to regenerate on the SFM upon allotransplantation (Krause et al., 1998). The resulting connections were found to generate larger EPSPs at around 25 mV than those evoked in the native SFM innervation which are normally less than 5 mV. Such synaptic properties are more indicative of the phasic system the transplanted nerve usually innervates as opposed to the tonic like innervation of the SFM system. Thus, from this experiment it appears that synaptic properties are ordained by the motoneuron and not the target muscle.

III. Aims of this study

During synaptogenesis the role of the neuron and/or its target in defining the structure of a synapse is still not clearly understood. While some developmental studies have been done, little work to date has examined these roles in adult systems. Genetic manipulations in embryonic Drosophila studies have shown that the neuron plays a pivotal role in active zone assembly (Prokop et al., 1996). However, this study did not examine the physiological properties nor the correlated structure of these neuromuscular synapses. As a result, the relative influences of neuron or its target in synapse structure and function could not be ascertained.

The SFM system of Procambarus clarkii provides an opportune platform for examining the roles of the neuron and its target muscle during synaptogenesis in an adult animal. This system has been well documented in its ability through transplantation to accept foreign innervation following denervation (Krause et al., 1998). Although a tonic system, the SFM has been found to accept phasic innervation. Such an ability allows for the comparison via
electrophysiology and fine structural analysis of novel synapses made on the muscle by a foreign transplant nerve to native synapses. If a mixed phasic-tonic nerve with a substantial number of motoneurons is used for the source of innervation, resulting synapses similar in nature to controls will suggest some retrograde influence or favouritism of the muscle in accepting tonic-like innervation. Conversely, if the resulting synapses appear to possess both tonic and phasic properties this is indicative of the motoneuron dictating synaptic phenotypes.
MATERIALS AND METHODS

I. Surgical Procedures

All crayfish used for these experiments were adult *Procambarus clarkii*, purchased from the Atchafalaya Supply Company of Louisiana. They were held communally in freshwater, aerated tanks at a temperature of 14°C. Host crayfish were first carefully denervated following previously established protocol (Ely and Vélez 1982) (Fig. 1). After being anaesthetized on ice, animals were secured in a wax-lined dish ventral side up and submerged in an ample supply of crayfish saline (van Harreveld, 1936). A small incision was cut close to the site of SFM nerve emergence from the central cord, then a glass hook was used to ease out the nerve whereupon it was cut with microscissors. Potential regeneration of the axotomized nerve was inhibited by cutting the nerve at its exit from the CNS, a process which greatly hampers any chance of regeneration (Hoy 1969).

Denervated animals were then allowed to recuperate for a period of 48 hours before undergoing allotransplantation.

The allotransplantation procedure used was one developed by Krause and Vélez (1995) ensuring the least amount of invasiveness and minimal formation of scar tissue. Donors were first sacrificed and the deep flexor and extensor muscles were removed so that the nerve cord would be exposed. The most caudal ganglion, ganglion 6, was then identified and a fine silk thread was tied rostrally to the ventral nerve cord. All roots emanating from the ganglion were cut close to their point of exit from the ganglion with the exception of the left (when viewed dorsally) third root, where a sizable remnant of its length was left. The cord was then transected just rostral to the previously tied silk thread and the ganglion along with its attached nerve cord tail was placed in cooled Ringer’s solution until the recipient
crayfish was ready. The inclusion of the ganglion ensured that the nerves along with their soma were transplanted.

The denervated host crayfish was once again anaesthetized on ice then placed in cooled crayfish saline ventral side up in a dissecting dish. A hole in the carapace was achieved by removing the left swimmeret of the third abdominal segment. A needle was then used to perforate the cuticle directly above the SFM creating a second much smaller hole. These two holes were connected by threading a human hair through both. This human hair would later serve to help guide the donor nerve into place. The end of the hair by the swimmeret hole was manipulated into a loop by tying a loose overhand knot and this loop was used to affix the cut end of the third root of the donor nerve after the donor ganglion was placed in the swimmeret hole. By gently pulling on the thread at the small hole the donor nerve could be manoeuvred into an intimate placement between the host muscle and ventral cuticle. Abutting the donor nerve to the host muscle is imperative if regeneration is to occur (Krause and Vélez 1995).

Altogether, 20 crayfish were allotransplanted with the donor nerve tissue to the denervated host SFM. The animals were held in individual compartments fashioned in 5 gal aquaria. Of these 20 transplant experimental animals 8 died, 4 moulted and the remaining 8 survived for 9-12 weeks during which time they were evaluated.

II. Electrophysiology

Animals were sacrificed for electrophysiology after a period of at least 10 weeks post-transplantation. This was due to earlier findings that it took at least 8 weeks for allotransplants to fully regenerate (Krause and Velez, 1995). Furthermore, this guaranteed that the distal portion of the transected native SFM nerve would be fully degenerated, a
process which takes around 3 weeks (Hoy et al., 1969; Ely and Vélez, 1982). Animals which had recently moulted were also dismissed as it has been observed that such animals exhibit synaptic repression perhaps a safeguard against deformity of the newly synthesized exoskeleton that would be caused by a fully functional SFM system (Gupta et al., 1993, Prosser et al., 1993). The abdomen was removed and the carapace along with the extensor and flexor muscles were dissected to expose the SFM and the nerve cord in the third abdominal segment being careful not to damage them. Crayfish in which any sign was observed that the native SFM nerve was not fully severed were discarded. The presence of a nerve over the SFM muscle that did not attach to the native nerve root as well as healthy appearing muscle fibres indicated that successful foreign innervation had taken place. Electrophysiology was then performed in Ringer’s solution at a temperature of 13-14°C. The foreign nerve situated over the muscle was stimulated using a suction electrode and a standard glass micro-electrode filled with potassium chloride was placed in various muscle fibres to record resting potentials of the muscle and any resulting EPSPs. The contralateral SFM with intact native innervation was used as a control.

III. Electron Microscopy
Following electrophysiological experiments, the SFM preparation were prepared for electron microscopy according to previously established procedures (Govind et al., 1994). The entire preparation consisting of both experimental and control SFM was fixed in situ with a primary fixative comprised of 2.5% glutaraldehyde, 0.5% formaldehyde, 1mM calcium chloride and 0.1M sodium cacodylate buffer (pH 7.4) for one hour at room temperature. Following this initial fixation, the SFM system in question along with its contralateral control were carefully removed and further fixed in an identical mixture for another hour. Three rinses of 0.1M sodium cacodylate in the course of an hour were used
to wash the tissue, then it was further fixed with a 2% osmium tetroxide solution in the 0.1M sodium cacodylate buffer for yet another hour. Once again the tissue was buffer-rinsed in 3 washes for 15 minutes and then dehydrated in a graded ethanol series. The tissue was cleared in propylene oxide for 30 minutes with 3 changes and then infiltrated overnight in a 50% propylene oxide, 50% Epon-Araldite mixture. This latter step helped the resin in thoroughly infiltrating the tissue. The next day the muscles and nerves were dissected in discrete bundles and placed in plastic embedding moulds filled with fresh 100% Epon-Araldite resin. The moulds were left at room temperature for a further 8 hours then cured in an oven at 60°C for 48 hours.

Thin sections (70 nm) of the tissue were obtained using a diamond knife on a Reichert OMU 2 Ultramicrotome. In the case of muscle, large areas (1x1 mm) were surveyed in order to increase the likelihood of finding nerve terminals and of surveying the full spectrum of motoneurons. When a nerve terminal region was located serial thin sections were obtained for approximately 10 μm. The colours of the thin sections caused by refraction as they float on water were noted individually. These colours aid in inferring section thickness with silver sections of approximately 70 nm being the most desirable. Ribbons, created by the adherence of each section to its predecessor were carefully picked up onto single-slot grids and laid on Formvar-coated slotted gridstands. Double staining with uranyl acetate and lead citrate completed the process whereupon the sections could be scrutinized with a Zeiss 9S electron microscope. Photographs of pertinent areas were then taken allowing for further analysis.

IV. Quantitative Analysis

Photographs of the serially thin sectioned nerve terminals were obtained with a final
magnification of x26,000 and these were used for quantitative analysis. Synaptic area was determined by measuring the length of synaptic profiles on the micrographs with calipers pre-set at 2 mm. These measurements were then multiplied by the thickness of their corresponding section; the thickness having been earlier estimated from inference colours during ultramicrotome sectioning. The addition of each calculated area from which the synapse was composed then gave the area of the synapse. Synapses that were for whatever reason not represented fully by the series of sections attained i.e. occurring at the end of a series or in a portion of a series that had been lost, were incorporated into a separate pool of data. In instances where isolated sections were lost, lengths of these particular axon profiles were estimated by averaging between the preceding and following sections. Mean synaptic area was determined by averaging out all the complete synapses for a given nerve terminal. Since a significant number of complete synapses were surveyed, the pool of incomplete synapses was disregarded in all further analysis. Determination of the length of tissue sectioned was calculated simply by adding the thicknesses of the individual sections cut in the series. It should be noted that terminal length was not calculated due to the complex nature of the regenerated terminal structure. Its non-linear appearance with numerous sprouts branching at all angles to the plane of section made such a determination difficult to measure.

Dense bars, a presynaptic specialization where transmitter release is proposed to occur, were located and noted on the micrographs. From this the number of dense bars present in a complete synapse could be ascertained and the mean density could be calculated by averaging the values of all complete synapses surveyed in a particular terminal. Frequency analysis of synapses containing 0, 1, 2 or 3 plus dense bars were then segregated to get a clearer picture of dense bar distribution. The number of dense bars per synaptic area was also calculated to give an indication of relative density.
Dense bar lengths were estimated according to their orientation upon being sectioned. Those that were transected longitudinally were measured directly on the micrographs using calipers and this length was then corrected with the appropriate magnification factors. If the dense bar was cut in cross section, simple addition of section thickness was used to estimate bar length. Again a mean was calculated by averaging all the dense bars occurring within a particular terminal.

Using some of the data collected above, two dimensional maps were created. This was accomplished by representing the length of each synaptic profile measured as a straight parallel line. Since section thickness typically was 70 nm, an appropriate space between subsequent lines was calculated and used. Field marks from the micrographs were used to help establish orientation of one synaptic profile to the next. Dense bars were represented as dots and shown in their relative position to the synaptic profile. If a dense bar spanned more than one section, successive dots were joined with a line. Branching and dead ends of the nerve terminal could also be represented according to their incidence in the series of sections.

Lastly, to get a better idea of the actual nerve terminal shape, three dimensional reconstructions were made. This was accomplished by tracing axon profiles directly from the micrographs and superimposing them so that each succeeding profile was shifted an appropriate distance to be indicative of the particular section thickness. The resulting picture gives a three-dimensional indication of what the nerve terminal actually looks like.
RESULTS

I. Physiological evidence for regeneration of neuromuscular synapses

Following the transplantation surgery, animals were examined approximately 10 weeks later. This time period was chosen as previous studies have shown that reinnervation by a crushed native or a foreign allotransplanted SFM nerve is achieved by 8 weeks (Ely and Vélez, 1982; Krause and Vélez, 1995). This time period also ensured that the decentralized nerve had degenerated, an occurrence that normally takes around 3 weeks (Hoy et al., 1969; Ely and Vélez, 1982). Out of the 20 transplant procedures performed, 8 of the animals survived. Each of these 8 were sacrificed then examined under the microscope to ascertain whether the native nerve had definitely been cut, as determined by the remainder of a stump off the cord, and subsequent reinnervation had taken place, as determined by the presence of a regenerate nerve on top of the muscle (Fig. 2). Only 4 of the 8 survivors showed both these prerequisites, and these were used for electrophysiological studies.

Measurement of 27 muscle fibre resting potentials showed normal values of 50-75 mV indicative of general health. Interestingly the regenerated nerve exhibited no spontaneous activity, a phenomenon always observed in a native tonic SFM nerve (Kennedy and Takeda, 1965b) as seen in the intact contralateral, control motoneuron (Fig. 3A). This lack of spontaneous activity provides further evidence that the experimental side had been completely disconnected from its native innervation. Despite this disconnection, the presence of functional synapses is established by the elicitation of EPSPs in all 4 preparations upon stimulation of the regenerate nerve (Figs. 3B-F). Of the 27 muscle fibres examined, 24 (89%) demonstrated synaptic potentials while 3 (11%) did not, denoting a lack of innervation. The majority, 18 of 24 received purely excitatory input, 2 received both excitatory and inhibitory input and 4 fibers received purely inhibitory input. Among the
Figure 2. Light microscope picture of the superficial flexor muscle following regeneration of a foreign nerve. The regenerate nerve can be seen lying over the muscle in an anterio-medial to postero-lateral direction. This nerve was stimulated and resulting postsynaptic potentials from the muscle were recorded. Bar: 1 mm. Magnification: x28.
Figure 3. Excitatory post-synaptic potentials (EPSPs) recorded from native (A) and regenerate (B-F) nerve terminals in the superficial flexor muscle. A, Spontaneous EPSPs characteristic of intact tonic innervation. B, Unitary EPSP demonstrating innervation via a single motoneuron. C, EPSP at 5 Hz stimulation showing little facilitation. D, Two EPSPs (arrows) indicating innervation by two axons. E, Three EPSPs (arrows) indicating innervation by three axons followed by an active response (double arrow). F, Twin pulse stimulation of a large EPSP (arrow) gives rise to an active response (double arrow) with the first stimulus, but not with a closely-spaced second stimulus which results in depression of the EPSP.

Vertical bar: A, D 10 mV; B, C, E 4 mV; F, 20 mV. Horizontal bar: A 20 msec; B 2 msec; C 100 msec; D, E, F 4 msec.
fibers innervated by the excitor axons, 8 received input from a single excitor (Fig. 3B), 8 from two excitors (Fig. 3D), and 4 from 3 excitors (Fig. 3E), as indicated by differences in the stimulus thresholds of the EPSPs.

EPSP amplitudes varied greatly ranging from 1-30 mV. The larger of these EPSPs often manifested in an active response (Fig. 3F). The simultaneous stimulation of 2 or 3 axons could also result in an active response at times (Fig. 3E). These action potentials were often observed to be followed by a twitch contraction of the muscle fibre. In the native tonic SFM preparations, a large EPSP eliciting an action potential is rarely seen (Kennedy and Takeda, 1965b).

To examine facilitation properties, regenerate nerves were repetitively stimulated. Native innervation of the SFM shows facilitation in all 5 of the excitatory axons which innervate it (Vélez and Wyman, 1976a). In the present study repetitive stimulation resulted in little to no facilitation (Fig. 3C). Cases where an active response was elicited failed to produce a subsequent action potential upon a second closely spaced stimulus and even showed signs of depression (Fig. 3F). Such properties are more indicative of responses seen in the phasic synapses of the DFM (Kennedy and Takeda, 1965a). While functional synapses were found to be present from the regenerate nerve, the properties of these connections possessed a greater variability and different properties from their native counterparts. They also differed from experiments where the SFM was allotransplanted as the resulting EPSPs in this case were usually less than 2 mV (Krause and Vélez, 1995).

II. Structure of regenerated foreign neuromuscular connections
In order to examine the structure of these functional foreign neuromuscular connections, following electrophysiology, the tissue in question was prepared for electron microscopy
along with its contralateral counterpart which acted as a control.

A. Muscle fibres

Cross sections of muscle taken on a plane parallel to the muscle fibres revealed sarcomere lengths to be relatively long ranging from 6 to 9 μm. These sarcomeres did not appear in register resulting in thick wavy Z-lines (Fig. 4A). Cross sections taken perpendicular to the length of the muscle reveal the number of actin filaments that typically surround a single myosin filament also known as the actin to myosin ratio. In this case 9-11 actin filaments surround a myosin filament (Fig. 4B). Both the sarcomere length and actin myosin ratios are typical of tonic type muscle (Atwood, 1976) and show that despite foreign innervation the muscle has retained its tonic identity.

An interesting aberration found in the foreign innervated muscle is the numerous appearances of myonuclei at the surface of the muscle surrounded by a thin band of granular sarcoplasm (Fig. 4C). Granular sarcoplasm is a specialized part of the muscle fibre capable of receiving innervation. In control muscle, granular sarcoplasm was never observed without at some point housing nerve terminals. Conversely, in experimental tissue these islands of nuclei with surrounding granular sarcoplasm were often found not to house any nerve terminals despite complete sectioning of their breadth.

B. Motor axons

Cross sections through the regenerate nerve found on top of the SFM and stimulated during the electrophysiological experiments showed 5 separate glial encased areas. Each separate glial encased area harboured several axon profiles, one relatively large one surrounded by many smaller ones (Fig. 5A). These smaller axon profiles are believed to be sprouts and have likewise been seen in other allotransplant experiments (Krause et al., 1996) and in the
Figure 4. Muscle fibres in allotransplanted preparation showing (A) in longitudinal section relatively long sarcomeres, delimited by Z-lines (2) and composed of an A-band (a) and I-bands (i), and (B) in cross-section 9-11 thin filaments around a thick one. (C) Muscle fibre (f) in allotransplanted preparation showing nucleus (n) with surrounding granular sarcoplasmic reticulum (re) devoid of nerve terminals.

Bars: A 5 μm; B 0.1 μm; C 1 μm. Magnification: A x64 800; B x117 300; C x24 400.
Figure 5. Cross-section of a regenerate nerve (A) showing five complex axons (arrows) each containing multiple axon profiles, and of a native nerve (B) showing six simple axons (arrows) each with a single profile. Cross-section of secondary branch of a regenerate nerve (C) showing four complex axons (arrows) and lying off-shore to the muscle. Intramuscular branch of a regenerate nerve (D) showing four axon profiles (arrows).

Bars: A 5 μm; B 0.1 μm; C 10 μm; D 2 μm. Magnification: A x64 800; B x117 300; C x2 500; D x5 800.
regeneration of severed motoneurons in crayfish limb muscles (Nordlander and Singer, 1972; Kennedy and Bittner, 1974). Conversely, cross-sections through the control nerve only show single axon profiles each surrounded by its own glial sheath (Fig. 5B).

This sprouting phenomenon is found along the length of the nerve as well as in secondary branches on the muscle surface (Fig. 5C). This indicates that sprouting continues throughout the regenerating nerve and into the muscle. Intramuscular branches of the control nerve (Fig. 5D) conversely give rise to nerve terminals that appear as single profiles.

C. Nerve terminals
To find areas with nerve terminal regions, muscles were surveyed by sectioning at predetermined intervals. Both the control and the reinnervated muscle showed the same frequency of innervation using this method. Following the muscle in this way offshore axons could be tracked to preterminal axon branches and finally to nerve terminal regions. Nerve terminals are decreed when motoneurons come into close proximity to the postsynaptic membrane so that a synaptic connection is possible. Many times in areas of regenerate terminals numerous small axon profiles are present continuing the pervasive branching trend that was also seen in lower order branches. The regenerate nerve terminals themselves are often very complex in nature, taking on a stellate-like shape in cross section with many finger-like projections all interconnected by very thin sheets of nerve (Fig. 6A). These intricate shapes contrast sharply with the control nerve terminals that tend to be round with only an occasional offshoot (Fig. 6B). Differences in their geometry are perhaps more fully realised when viewing three dimensional reconstructions. Regenerate nerve terminals have many little offshoots that protrude in all directions both parallel and perpendicular to the cross section (Fig. 7B, C). Sometimes finger-like projections of granular sarcoplasm
Figure 6. Regenerate (A) and native (B) nerve terminals (t) are defined by predominantly clear synaptic vesicles and well-defined synaptic contacts (between arrows) made adjacent to muscle sarcoplasmic reticulum (re). Some synapses show a presynaptic dense body (arrowheads). A few mitochondria and dense core vesicles are present. Regenerate nerve terminal (A) shows several interconnected branches which arise from intramuscular axon branches (a) compared to native terminals (B) which consist of single branches.

Bars: 0.1μm. Magnification: A x25 400; B x35 400.
Figure 7. Three-dimensional reconstruction of native (A) and regenerate (B, C) nerve terminals showing the simpler nature of the former compared to the complex, branched nature of the latter.

Bars: 2 μm.
are seen to penetrate into the nerve terminal appearing in cross section as islands of granular sarcoplasm in the middle of the nerve profile. It is unknown whether these projections arise due to the nerve growing around the granular sarcoplasm structure or are a result of the granular sarcoplasm growing into the nerve terminal, although the former seems more plausible. The control nerve terminals are far more linear in fashion (Fig. 7A). They have occasional branches, but unlike their regenerate counterparts these branches tend to run parallel to their precursors. Both control and regenerate terminals contain a large population of clear synaptic vesicles, a smaller number of dense-core vesicles and mitochondria (Figs. 6A, B).

D. Synapses

Synapses are sites of neuromuscular connection and are denoted structurally by the close apposition of the pre and post-synaptic membranes (Figs. 6A, B). In synaptic areas, these membranes become more electron dense and are smoother in appearance as they run directly parallel to each other. They are separated by a 15 nm synaptic cleft which is filled with electron dense material. Once terminal regions were located, they were serially sectioned so that their respective synapses could be analyzed and compared. In total, 20 of these terminal regions were examined; 12 regions from regenerate tissue and 8 regions from the control tissue comprising 248 and 185 complete synapses respectively (Table 1).

Analysis of synaptic areas shows a greater variation in the regenerated nerves than in native SFM innervation (Fig. 8). While on average the regenerate synapses are significantly bigger at $0.448 \pm 0.380 \, \mu m^2$ compared with $0.336 \pm 0.197 \, \mu m^2$ for the native ($p<0.0005$) (Table 1), perhaps a more illuminating perspective comes from looking at the values of individual terminals (Fig. 8). This reveals that regenerate synapses in one terminal
Table 1. Quantitative analysis of synapses and dense bars in regenerate and native nerve terminals to the crayfish abdominal superficial flexor muscle

<table>
<thead>
<tr>
<th></th>
<th>Regenerate</th>
<th>Native</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of terminals</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td><strong>Synapses</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>number</td>
<td>248</td>
<td>185</td>
</tr>
<tr>
<td>mean area ($\mu m^2$)</td>
<td>$0.448 \pm 0.380$</td>
<td>$0.336 \pm 0.197$</td>
</tr>
<tr>
<td></td>
<td>(<em>p</em>≤0.0005)</td>
<td></td>
</tr>
<tr>
<td>total area ($\mu m^2$)</td>
<td>111.207</td>
<td>62.232</td>
</tr>
<tr>
<td><strong>Dense bars</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>number</td>
<td>562</td>
<td>317</td>
</tr>
<tr>
<td>number / synapse</td>
<td>2.3 ± 1.9</td>
<td>1.5 ± 1.0</td>
</tr>
<tr>
<td>number / synaptic area</td>
<td>5.1</td>
<td>4.4</td>
</tr>
<tr>
<td>mean length ($\mu m$)</td>
<td>$0.123 \pm 0.055$</td>
<td>$0.103 \pm 0.041$</td>
</tr>
<tr>
<td></td>
<td>(<em>p</em>≤0.0001)</td>
<td></td>
</tr>
<tr>
<td>length / synaptic area ($\mu m$)</td>
<td>0.743</td>
<td>0.526</td>
</tr>
<tr>
<td>% synapses with:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 dense bar</td>
<td>6.9</td>
<td>15.7</td>
</tr>
<tr>
<td>1 dense bar</td>
<td>36.7</td>
<td>43.2</td>
</tr>
<tr>
<td>2 dense bars</td>
<td>23.8</td>
<td>27.6</td>
</tr>
<tr>
<td>3+ dense bars</td>
<td>32.6</td>
<td>13.5</td>
</tr>
</tbody>
</table>
Figure 8. Histogram showing rank order of mean synaptic size in 8 native (A) and 12 regenerate (B) nerve terminals. Regenerate synapses are seen to have a greater size range than their native counterparts.
population have an average size as low as 0.140 ± 0.065 \( \mu \text{m}^2 \) compared to the largest terminal population at 0.701 ± 0.354 \( \mu \text{m}^2 \). This covers a much greater range than native SFM synapses whose individual terminal populations have a low extreme of 0.202 ± 0.090 \( \mu \text{m}^2 \) and a high of 0.525 ± 0.279 \( \mu \text{m}^2 \). These differences in synapse size are also apparent in two dimensional mapping of the nerve terminals (Fig. 9). Figures 9B, C show representations of the regenerate nerve terminals possessing some extreme synaptic areas compared to Figure 9A which shows an average native innervation situation. Again a much greater range in synaptic area for the regenerate nerve is evident.

E. Presynaptic dense bars

Structurally presynaptic dense bars represent active zones which physiologically are the sites of neurotransmitter release (Heuser et al., 1979). Qualitatively these dense bars are found to be similar in both the regenerated and native motoneurons. They are the arena for vesicle docking and release and depending on what plane they are sectioned take on the respective appearance of a distinctive shape. Cross-sectionally they appear T-shaped with vesicles docked on either side of the vertical strut (Fig 10 A, C). Longitudinal sectioning results in a bar-shape presynaptic dense body with vesicles docked along the long axis of the bar (Fig. 10B, D). Sometimes the exocytosis process can be caught as indicated by the appearance of a fused vesicle adjacent to a dense bar (Fig. 10E), the dense bar in this case being located in the previous section. Many times a cap will appear as an arc over the active zone seeming to help anchor it down (Fig. 10F). The precise function of this cap is unknown.

Although qualitatively these dense bars appear the same between native and regenerate terminals, when measurements are taken of their length a difference is uncovered. A hint
Figure 9. Two-dimensional reconstruction of a native (A) and two regenerate (B, C) nerve terminals showing the size and shape of synapses as thin vertical lines, and the number and length of presynaptic dense bodies as thick horizontal lines. Regenerate synapses in B, C are distinguished from native ones in A by their greater size range and by their longer and or more numerous dense bars. 

Bars: 0.1 μm.
Figure 10. Nerve terminals (t) from native (A, B) and regenerate (C-F) motor axons showing synaptic contacts (between arrows) adjacent to muscle granular sarcoplasmic reticulum (re). Presynaptic dense bars (arrowheads) in control synapses are usually single (A) and short (B), while in regenerate synapses they occur in multiples (C) and are much longer (D). Also in regenerate axons (E) and nerve terminals (F) presynaptic dense bars with clustered synaptic vesicles (double arrowheads) are found in non-synaptic location adjacent to glial (g) tissue, in addition to their usual location (arrowheads) at synaptic contacts. Note exocytotic vesicle (arrowhead) in (E) and cap (arrowheads) for pre-synaptic dense bars in (F).

Bars: A-D 0.1 μm; E, F 0.2 μm. Magnification: A-D x117 100; E x85 000; F x67 400
of this difference is suggested by visual inspection of longitudinal sections taken of
regenerated and native active zones (Fig. 10B, D) in which the regenerated synapses clearly
bear longer active zones than their native counterparts. This prediction is corroborated by
results of active zone length measurements. Plotting these measurements for each terminal
surveyed against their relative synaptic area (Fig. 11A) reveals that the regenerate active
zones were longer than the native ones. One of the regenerated terminals possessed the
longest active zones. The tendency for longer active zones in regenerated terminals is also
seen in the overall average measurements (Table 1). The regenerated active zones are
significantly longer than their native counterparts (p ≤ 0.0001). Longer active zones would
increase the efficacy of regenerate nerves by allowing more vesicles to dock.

Distribution of active zones within the synapses was also considered in one of two ways.
First, the number of dense bars per μm² of synapse was calculated and proven to be higher
(5.1 dense bars per μm² of synapse) for the regenerate terminals than in native terminals
(4.4 dense bars per μm² of synapse) (Table 1). Second, and more striking was differences
found in the number of dense bars per synapse between regenerate and native terminals.
Regenerate foreign nerve terminals on average are found to contain more active zones per
synapse at a mean of 2.3 ± 1.9 dense bars per synapse compared to a native value of 1.5 ±
1.0 dense bars per synapse (Table 1). Looking at values for individual terminals again
provides further information. Of the twelve regenerate terminals surveyed, eight have on
average 1 to 2 dense bars per synapse much as what is seen in native terminals (Fig. 11B).
However, 4 of the twelve have an average of 3 to 4 dense bars per synapse. Taken
collectively 32.6% of all synapses measured in the foreign regenerate terminals have 3 or
more dense bars compared to only 13.5% of all the native terminals (Table 1). Clearly some
foreign regenerate populations have many more active zones per synapse.
Figure 11. (A) Plot of the relationship between synapse size and dense bar length showing that dense bars are longer in regenerate than in native synapses. Equation of the line for regenerate terminals: \( y = 0.088 + 0.014/x \), \((r = 0.80)\). Equation of the line for native terminals: \( y = 0.085 + 0.006/x \), \((r = 0.46)\).

(B) Plot of the relationship between synapse size and number of dense bars per synapse showing that regenerate synapses have more dense bars per synapse than native synapses. Equation of the line for regenerate terminals: \( y = 0.618 + 3.808x \), \((r = 0.82)\). Equation of the line for native terminals: \( y = 1.445 + 0.007x \), \((r = 0.003)\).
By plotting the mean number of dense bars per synapse against the mean synaptic area for the individual terminals one can further corroborate the above impression of a higher density for regenerate than native terminals (Fig. 11B). An ectopic group of terminals with greater synaptic areas and a higher number of dense bars present per synapse become evident in the regenerate tissue. Thus two clearly distinct populations of nerve terminals in addition to that normally seen in the native SFM innervation are apparent in the regenerated foreign connections. The first, bearing synapses typified by a higher synaptic area with a greater number of active zones and the second, by a smaller synaptic area with less and longer active zones.

Regenerate foreign nerve terminals were also found to have many extrasynaptic dense bars. These dense bars look very much like their neuromuscular counterparts but tended to lie in apposition to glial cells instead of granular sarcoplasm (Fig. 10E, F). Such extrasynaptic placement of dense bars was not noted in native neuromuscular tissue.

F. Inhibitory innervation

Although not specifically analyzed in this case it should be noted that inhibitory innervation was found in both native and regenerated terminals. Inhibitory terminals are distinguished from their excitatory counterparts by some irregular, elliptically shaped vesicles, a narrower synaptic cleft and less electron dense presynaptic and postsynaptic membranes (Atwood, 1976). As no previous fine structural study of this preparation has been undertaken, examination of the native terminals via serial thin sectioning definitively revealed that in addition to direct inhibitory connections with the muscle, presynaptic inhibition does indeed exist in both medial and lateral portions of the SFM as illustrated by a series of micrographs showing an axo-axonal synapse with the active zone on the inhibitory side (Fig. 12). While
such connections were believed to occur in the SFM as a result of pharmacological studies whereby the relative positions of inhibitory and excitatory neurotransmitter sensitivities were localized (Takeuchi and Takeuchi, 1965), this is the first direct evidence that inhibitory axo-axonal connections are present. In comparison to the frequency of inhibitory connections made directly onto the muscle, presynaptic inhibition occurs far more sparsely.
Figure 12. (A-F) Serial micrographs of an inhibitory (i) nerve terminal making axo-axonal synaptic contacts (between arrows in C, F) with an excitatory (e) terminal denoting presynaptic inhibition. The direction of transmission is indicated by the aggregation of synaptic vesicles and the occurrence of a presynaptic dense bar (arrowhead in C) on the inhibitory side. Identification of the inhibitory terminal was based on a long series of micrographs which showed some irregular shaped clear synaptic vesicles, and neuromuscular contacts with narrower clefts and less intense staining.

Bar: 1 μm. Magnification: x23 000.
DISCUSSION

Transplantation studies in the past have been used to assess genetic versus epigenetic factors of innervation in locusts (Anderson, 1985), to assess retrograde influence on central and peripheral connections in leeches (Loer and Kristan, 1989), to restore simple behaviours in snails (Syed et al., 1990) and to examine central connections of sensory nerves in crickets (Murphey and Chiba, 1990; Chiba et al., 1988). This study endeavours to use a foreign transplanted nerve made up of both tonic axons, which are indigenous to the intended target, as well as phasic axons which are foreign, to assess the fastidiousness of the muscle in accepting innervation and the role played by the neuron and muscle in synaptogenesis.

The third root of the crayfish sixth segment along with its associated sixth ganglion was allotransplanted to the SFM of the third segment. This muscle system is tonic in nature contrary to the mixed phasic and tonic motoneurons that are present in the nerve root transplanted (Higuchi, 1992). Through electrophysiological recordings, viable synaptic connections were found to occur. The ability of a foreign neuron to successfully innervate a mismatched target upon transplantation or misrouting techniques have been found in a number of preparations. Vertebrate muscle has long been known to readily accept foreign innervation (Elsberg, 1917; Frank and Jansen, 1976), but only more recently has it also been noted in invertebrates. Insect motoneurons were found to successfully innervate foreign muscle targets following the removal of their normal targets (Nässel et al., 1986; Whittington and Seifert, 1984; Whittington, 1985). Ablation of targets in Drosophila embryos by genetic and laser methods has resulted in ectopic connections made by developing motoneurons (Cash et al., 1992). Using the same tonic SFM preparation utilized in this study, transplants of the phasic nerve that normally innervates the deep flexor muscle (DFM) were further found to make viable connections (Krause et al.,
1998). In conjunction with the findings listed above, the success of foreign transplants in this study suggest that common properties involved in synaptogenesis must be present in the nerve and muscle.

Analysis of electrophysiological recordings and the fine structure possessed by the resulting ectopic synapses made by the transplanted foreign nerve in this study show a wide variation in findings. While some connections clearly resemble those indigenous to the target muscle, synapses with outlying properties are also present. The divergent properties observed include much greater excitatory post synaptic potentials with little to no facilitation, mean synaptic areas above and below native parameters, much longer dense bars and a greater mean number of dense bars per synapse. The existence of these anomalous synapses along with the finding that the integrity of the muscle properties remain intact, implicates the nerve as being solely responsible for synaptic phenotypes and resulting synaptic properties. The muscle appears to show no favouritism for one nerve type over the other, although it does seem to have a lower than normal carrying capacity as muscle fibres had no more than three motoneurons compared to the native fibers possessing up to five motoneurons (Kennedy and Takeda, 1965b).

I. Induction of nerve sprouting and neural guidance
Invertebrate neurons have been observed to sprout and regrow following a variety of traumas. Arthropod nerves that have been damaged in the CNS responded through sprouting and regrowth (Fredman and Nutz, 1988; French and Muller, 1986). Likewise damage to leech axons in conjunction with the removal of their target, or the availability of a suitable target, resulted in sprouting of these axons (Bray et al., 1972). Structural analysis in crayfish showed extensive sprouting through the presence of multiple axon profiles of a regenerating opener muscle nerve 12 weeks post lesion (Nordlander and Singer, 1971;
Kennedy and Bittner, 1974). Analogous sprouting has also been observed in the present allotransplantation experiments, as well as allotransplantation studies done previously (Krause et al., 1996; Krause et al., 1998).

What triggers sprouting in these axotomized nerves? The signal for sprouting induction can either come from the nerve itself in response to injury, from the presence of a denervated target or simply from the loss of a target in general. The last of these possibilities has been suggested to be implemented whenever there is a drop in the availability of a trophic factor normally supplied by the now absent muscles, signaling the nerve cell body to initiate sprouting (Cragg, 1970; Dennis, 1981). Factors originating from muscle cells have been observed to exert a clear positive affect on nerve sprouting (Gurney, 1984; McCaig, 1986). They include both membrane bound substances such as N-CAM (Covault et al, 1986) and N-cadherin (Bixby and Zhong, 1990) as well as diffusible substances such as a cytokine growth factor by the name of pleiotrophin (Li et al., 1990, Raulo et al., 1992). It has been hypothesised that these environmental cues affect growth cone motility by somehow modulating the calcium concentration inside the growth cone (Patel and Poo, 1984; Lockerbie, 1987; Rees et al., 1976). It would follow that a severed axon could undergo growth cone differentiation and consequently would also be susceptible to these same environmental cues. Since the allotransplanted nerve successfully grows towards the denervated target, it is probable that some of these environmental signals are indeed diffusible signals arising from the muscle. The existence of a signal for neurite outgrowth being present in the muscle was substantiated in studies of the Drosophila mutant twi which fails to undergo muscle development. Consequently without the presence of muscle cells, axons failed to migrate (Prokop et al., 1996).

The fact that regeneration from foreign allotransplants to the crayfish SFM (Krause et al.,
1998; present report) as well as native transplants to the SFM (Krause and Vélez, 1995; Krause et al., 1996) is possible, lends credence to the opinion that the diffusible signal the muscle releases upon denervation must be common in nature. The ubiquity of this signal is also suggested in the ability of neurons to make contacts with adjacent muscle targets as a result of ablation of their target through genetic and laser methods (Cash et al., 1992). However, despite the loss of their target these developing neurons are still able to migrate to their appropriate area in the body wall. This finding shows that at least during development, other environmental cues are also responsible for neuronal targeting in addition to the apparent common muscle derived cues. In regenerating cases it has also been postulated that denervated muscle via some unknown factor, can bring about the accumulation of certain interstitial cells in the connective tissue spaces of abandoned synaptic sites which effectively makes the area of accumulation better suited to motoneuron outgrowth (Connor and McMahan, 1987; Gatchalian et al., 1989).

Whatever the impetus for axonal sprouting the sheer quickness with which sprouting has been observed suggests that control mechanisms responsible for sprouting may be present in the axon tip. The relative slowness of axonal transport would require substantially more time to communicate with the nerve cell body than the time observed for the onset of outgrowth (Bray et al., 1972). This would indicate that sprouting is initiated without the involvement of the cell body. Furthermore, axons that have been disconnected form their somas have differentiated a new growth cone at the site of decussation (Mason and Muller, 1982). This definitively implies that many of the mechanisms essential to nerve sprouting are present throughout the axon and not dependent on the soma.

II. Neuronal specification of regenerated synaptic properties

Upon successful innervation of the tonic host target by the foreign transplanted mixed
nerve, electrophysiological and structural analyses were performed. Electrophysiology showed excitatory post synaptic potentials that ranged from 1 to 30 mV. The smaller of these potentials resemble the tonic responses seen in the native SFM preparation. However, the larger of these potentials were often seen to be accompanied by an active response and displayed depressive properties in response to repetitive stimulation. Such properties are more reminiscent of those seen in the phasic deep flexor muscle system (Kennedy and Takeda, 1965a). It appears that both tonic and phasic neurons present in the allotransplanted nerve have successfully made synaptic connections.

It should be noted that the degree of facilitation of the smaller EPSPs seen in the transplanted system was less than that normally exhibited by the native axons. This is probably a symptom of a less rigorous trophic programme being available to the transplanted nerve. Decreased trophic support would likely result in a decreased ability of the neuron to synthesize and recycle synaptic vesicles culminating in a more exhaustive store of synaptic vesicles. Short-term facilitation is the phenomenon where a greater release of neurotransmitter is evoked by repetitive stimulation resulting in progressively greater EPSP amplitudes (Atwood, 1976). It is a presynaptic event thought to be due to an increase in calcium concentration levels due to prolonged depolarization of the terminal (Atwood et al., 1987). The residual calcium hypothesis, as it has become to be known, suggests that the build-up of residual calcium along with the fourth power relationship of calcium to neurotransmitter release (Augustine and Charlton, 1986), results in a non linear increase in the chance of quantal release (Atwood and Wojtowicz, 1986). Another possible mechanism responsible for short-term facilitation is the occurrence of phosphorylation during neurotransmitter release (Swain et al., 1991). While exocytosis has been found to be regulated by calcium binding (Atwood, 1976; Zucker, 1987), phosphorylation in part has been found to regulate the slower process of vesicle mobilization. If phosphorylation
does play a role in short-term facilitation, the use of phosphatase inhibitors would be expected to adversely affect facilitation levels. Treatment with a phosphatase inhibitor was indeed found to reduce facilitation levels (Swain et al., 1991). Since the phosphatase inhibitor was not found to alter internal calcium concentration levels, this clearly implicated phosphorylation as part of the mechanism responsible for short-term facilitation. Due to the lesser degree of activity in the transplanted nerve, activity dependent kinases and phosphatases found in the transplanted nerve, may be attenuated. This by its own, but more likely in conjunction with decreased stores of vesicles due to less accessibility of the transplanted nerve to trophic factors, is likely responsible for the decreased facilitation levels observed.

Structural analysis corroborated the presence of atypical synaptic connections on the host muscle. While once again the presence of synapses lying within the range of that set out by native SFM innervation are apparent, there are also distinct populations of outlying synapses. These novel synapses include populations of much larger than normal synapses with the inclusion of many dense bars per synapse as well as smaller than normal synapses with considerably longer dense bars. The dense bars measured in the control populations are all uniform in length while in one population of regenerated terminals a propensity for much longer dense bars, typically spanning 3 or more thin serial sections, is observed.

Longer dense bars are not only capable of docking and releasing a greater number of vesicles but are also thought to increase the likelihood of transmitter release due to the possession of more calcium channels (Walrond et al., 1993). Calcium channels each exert their own microdomains which is the area directly surrounding the channel opening that a resultant gradient of calcium concentration exists as a result of the opening (Augustine et al., 1991; Llinás et al., 1992). The presence of more calcium channels in a longer dense bar
would result in greater overlapping of their microdomains. This would positively affect the local calcium concentration at the active zone and greatly increase the chance of neurotransmitter release resulting in greater EPSPs. Likewise, a greater number of dense bars available to a synapse, also known as the binomial parameter n (Atwood and Cooper 1996), have also been associated with greater transmitter release (Atwood and Marin, 1983) resulting in greater EPSPs. Consequently, it appears that these novel foreign synapses in terms of differing structural characteristics are most likely the same ones exhibiting the atypically larger depressing EPSPs. Synapse structures containing both longer and more complex dense bars have been found to be characteristic of phasic motoneurons (King et al. 1996). Thus phasic and tonic axons appear to be innervating the muscle. The ability of both phasic and tonic axons making connections with this inherently tonic muscle not only reveals the nondiscriminating acceptance of the muscle but also implicates the nerve in mandating the structure and functional properties of the resulting synapses.

This finding is corroborated in vertebrates where the release of ACh from growth cones cultured in the absence of muscle suggests that much of the requirements needed for the synthesis, packaging and release of neurotransmitter are independent of nerve-muscle contact (Hume et al., 1983; Young and Poo, 1983). This would tend to negate a muscle derived factor being the trigger, but rather inferring that a considerable portion of the apparatus required for neurotransmitter release must be the result of an autonomous program of the nerve. Further evidence for the neuronal autonomy responsible for this apparatic assembly has come from studies on genetically engineered Drosophila. A series of mutants resulting in different degrees of muscle development were used to assess the influence of the post synaptic cell on synaptogenesis (Prokop et al., 1996). Even the most severely affected of these mutants, the twi mutant in which no development of muscle occurs, was capable of successfully developing active zones showing that in the Drosophila
developing system the assembly of active zones is a completely independent process inherent to the presynaptic neuron. The trigger responsible for the assembly of active zones is still unknown. The ability of viable active zones to form in syntaxin *Drosophila* mutants in which all neurotransmitter release is blocked, dismisses the trigger from being a response of the neuron to its own neurotransmitter release (Broadie et al., 1995).

The neuronal autonomy suggested by this experiment in establishing synaptic structure along with the resulting functional synaptic properties seems to be contradictory to previous indications of retrograde influences. We did not look at possible differences in synaptic properties of the same axon on differing muscle fibre targets. Such differences have been detected in the crayfish opener muscle where although originating from the same motoneuron, proximal muscle fibres support high output synapses in contradistinction to low output synapses supported by distal fibres (Atwood and Bittner, 1971). This would suggest some signal from either the proximal or distal muscle is at work causing one of the synaptic phenotypes to be altered. Similar signals from the target as a whole have also been implicated in causing different synaptic properties within the same excitatory axon that contacts two separate targets (Davis and Murphey, 1993; Katz et al., 1993). Perhaps the best studied of these systems is that seen in the cricket. Mechanosensory neurons from the cerci are found to make facilitating connections on one motoneuron (MGI) and depressing connections on another (10-7) (Davis and Murphey, 1993). These researchers believe that the depressing synapses are the default state while the facilitating synapses are a result of a retrograde signal from the MGI interneuron. It is quite possible that while all axons have an inherent synaptic phenotype dictated by their autonomous program, they are subject to a genetically defined range of properties when influenced by either a retrograde signal or differing activity demands. It would be more adaptive for an organism to evolve a retrograde signal from the post-synaptic cell than to develop a whole new presynaptic cell to
meet activity requirements. A molecule has been recently isolated in *Drosophila* that may act as one of these retrograde signals (Davis and Goodman, 1998). This could possibly shed some light on the mechanism by which synaptic properties can be chemically altered. Transplanted neurons in our experiments failed to exhibit spontaneous activity so if a muscle's retrograde signal is released in response to neuronal activity, perhaps the release of retrograde signals were thwarted as a result of this lowered activity. However, it is more likely that targets innervated with uniform synaptic properties that don't share their neurons with other targets possessing nonuniform properties, lack retrograde influences instrumental in determining synaptic structure and properties. The requirement for a retrograde signal would be redundant in such cases.

Activity may also have a modelling effect on these default synapses. The imposition of repetitive stimulation on a developing phasic motoneuron in a juvenile crayfish, successfully altered its properties to that of a more tonic one (Lnenicka et al., 1986, 1991). These changes included reduced levels of initial transmitter release, reduced fatigability and associated changes in fine structure including an increased number of varicosities and greater mitochondrial content persisting for at least 10 days. Since this conditioning regimen of stimulation was still able to induce these changes when applied centrally, it was clear that the alteration in phenotype was a result of neuronal impulse activity as opposed to muscle or neuromuscular synaptic activity (Lnenicka and Atwood, 1989). This showed that neuronal activity may be a factor in the development of adult synaptic properties. In our neuronal mismatch experiment we were dealing with an adult system, and while the imposed activity experiments outlined above were successful in young crayfish, this success did not carry over equally into adult specimens (Lnenicka and Atwood, 1985). It is possible in the present experiments that regenerating axons may reacquire some of the plastic capabilities they possessed while young, and thus be susceptible to neuronal activity
helping to mould the appropriate synaptic phenotype. However, remembering that the transplanted neurons in our study failed to show spontaneous activity, suggests that neuronal activity was not a prime factor in shaping synaptic properties during regeneration. Synaptic properties made by neurons are the result of an autonomous program, but whether their properties may later be altered by neuronal activity is unresolved.

The presence of ectopic dense bars in the regenerating neurons that don’t occur at sites of neuromuscular contact lend further credence to the hypothesis that nerves are responsible for the assembly of active zones independently of the muscle. Many regularly structured dense bars were found in the regenerate nerve to lie in apposition to glial tissue. This suggests that the manufacturing of these dense bars preceded neuromuscular contact. It is probable that with time most of these ectopic dense bars would regress due to their lack of proximity to the muscle membrane, which could serve to consolidate synaptic connections.

III. Target acceptance of foreign regenerating axons
The crayfish target muscle in this experiment showed no discretion, equally accepting both phasic and tonic axons, a tentative identification based on synaptic fine structure. Although mapping studies of the SFM’s native innervation showed a gradation of synapses as measured by EPSP amplitudes across the muscle’s surface from a medial to lateral direction (Vélez and Wyman, 1978a), no such trends were seen here. Environmental cues have been hypothesized to be responsible for the above trend found in the muscle’s native innervation and are believed not only to be present during development but throughout the animal’s life as connectivity maps are reinstated following reinnervation (Ely and Vélez, 1982). Perhaps the effects of these proposed cues’ are lost on these foreign nerves being specific to the SFM nerve. Possibly the trend in innervation is genetically coded by the SFM nerve, but this seems unlikely as reduced target sizes and ectopic placements of the
nerve in regeneration studies have still resulted in the same innervation trends (Clement et al., 1983; Goransson et al., 1987; Hunt and Vélez, 1989a; Worden et al., 1987). The finding that each SFM fibre is innervated by one proctolinergic motoneuron and one non-proctolinergic motoneuron (Bishop et al., 1987) suggests that postsynaptic cues may be sensitive to the presence of a co-transmitter. The regenerated terminals in our experiment contained more than one kind of transmitter, indicated by the presence of clear as well as several sizes of dense core vesicles. If a cue sensitive to specific co-transmitters exists, perhaps this cue vetoed the more subtle cues for gradation in synaptic properties. Whatever the reason, favouritism by the muscle for one type of innervation over the other was not observed in these allotransplantation experiments.

In studies performed on Drosophila afflicted by muscle development mutations, two steps were defined in the process of synaptogenesis (Prokop et al., 1996). The first of these steps as discussed above, involves the assembly of active zones, sculpted by a genetic programme autonomous to the presynaptic membrane. The second of these steps involves the postsynaptic cell and its role in localizing the developing synapses. In a mutant which fails to undergo myoblast fusion (mbc), 45% of active zones were found to be in positions adjacent to the muscle while the other 55% were extrasynaptic. In a more severely affected mutant, mef2, in which β3-tubulin and myosin expression are neutralized resulting in abnormally structured myofilaments and incomplete muscle attachment, no instances of active zones being localized to the muscle are seen although the neurite still grows out to the muscle surface. Thus something present in the mbc mutant but lacking in the mef2 mutant plays a role in the localization process. Vertebrate neurons grown in culture where no muscle remained except for its basal lamina sheath, were successfully found to make connections localized on the basal lamina at congruous locations to former synaptic sites. (Glicksman and Sanes, 1983; Rich and Lichtman, 1989; Sanes et al., 1978; Yao 1988).
Thus, in vertebrates the substance responsible for active zone localization must be located in the basal lamina. A substance, s-laminin, has recently been isolated and is thought to be instrumental in this role (Guatam et al., 1996; Noakes et al., 1995; Sanes et al., 1978). However, while nerve terminal differentiation and synaptogenesis was possible in the sole presence of the basal lamina, these connections were only transitory lasting for a few weeks (Sanes et al., 1978; Yao, 1988). This suggests that substances originating within the muscle are necessary to sustain and consolidate synapses. Supporting this conclusion was the observation that stability of synapses on the basal lamina could be evoked if the cultured neurons were exposed for as little as 3 days to muscle fibres (Yao, 1988). The absence of active zones found in regenerating neurons cultured on polypeptide coated latex beads despite nerve terminal differentiation (Peng et al., 1987) further implicates the muscle as serving in a consolidating capacity.

Much work, predominantly on vertebrates has been done to try and ascertain the mechanism by which muscle is able to consolidate synapses. Although only pieces of the puzzle have been unfurled, it is probable that the process is a rather complex one, perhaps triggered in response to signals from the outgrowing neuron resulting in a relay of events between the nerve and muscle. One candidate for the possible presynaptic trigger is the release of neurotransmitter. In vertebrates, ACh is capable of being released in the absence of muscle (Hume et al., 1983; Young and Poo, 1983). This independent release could jump start muscle activity, conceivably through a second messenger system, which has been established to be instrumental in regulating muscle metabolism. This metabolism could in turn result in the expression and stabilization of proteins vital to synaptogenesis (Laufer and Changeaux, 1989; Sanes and Lawrence, 1983). Although the assembly of glutamate receptors in insect neuromuscular junctions is autonomous to the muscle, the concentration of these receptors has been found to be dependent on motoneuronal interaction (Broadie
and Bate, 1993; Currie et al., 1995). Possibly the release of glutamate from the growing neurons is also responsible for receptor aggregation. Agrin, a substance released from the motoneuron in vertebrates, stimulates acetylcholine receptor aggregation (Bowe and Fallon, 1995). Thus it could be equally plausible that a parallel substance, instead or in conjunction with glutamate, is released from invertebrate motoneurons inducing receptor aggregation.

Further interaction between nerve and muscle are believed to be initiated after neuromuscular contact. Through measurements of synaptic currents in the muscle, neuromuscular contact has been found to result in even greater ACh release from the motoneuron (Chow and Poo, 1985; Evers et al., 1989; Xie and Poo, 1986). This increased neurotransmitter release is due to contact as the same phenomenon is observed in muscles that have been turned outside-out (Xie and Poo, 1986). Presumably the greater release of neurotransmitter is a consequence of increased resting calcium concentrations in the nerve terminal (Funte and Haydon, 1993; Zoran et al., 1993). The source for the increase of calcium levels could be from extracellular calcium through the initiation of calcium channels, or from the purging of calcium stores within the terminal itself. Evidence that channels are probably involved come from studies where decreased extracellular calcium result in decreased internal resting calcium levels due to neuromuscular contact (Dai and Peng, 1993). Whatever the source for the contact-induced increase in intracellular calcium, it seems to be mediated by the activity of a cAMP dependent protein kinase (Funte and Haydon, 1993).

Synaptic activity appears to play an inductive role in synaptic refinement. The initial rise in calcium concentration due to neuromuscular contact induces even more calcium to be present in the terminal. The removal of extracellular calcium in cultured motoneurons
during synaptogenesis, results in disturbances to terminal differentiation. While functional synapses are still made, they were found to exert smaller junctional potentials than normal (Holliday and Spitzer, 1990). In our experiment no discernible spontaneous activity was recorded prior to stimulation. This would indicate that levels of activity in these transplanted motoneurons are below normal. However, the resulting connections evoked junctional potentials that were in some cases much greater than normal. Perhaps a higher level of activity would have resulted in more uniform synaptic phenotypes that were more tonic in nature. Contradicting this conjecture are findings from allotransplantations of the SFM nerve from one animal into a corresponding denervated SFM preparation in a host animal where the resulting connections had weaker potentials (Krause and Vélez, 1995). Together, the success of allotransplantation studies in general do show that little neuronal activity is required to consolidate new synaptic connections. Remembering that imposed activity had a much diminished affect on synaptic plasticity in adults (Lnenicka and Atwood, 1989, but see Mercier and Atwood, 1989), the lack of activity in allotransplantations probably results in a minimal difference. If anything, higher activity levels may have resulted in relatively insignificant refinements to these synapses.

The curious phenomenon of myonuclei with surrounding uninnervated granular sarcoplasm was found solely in regenerated tissue raising a couple of possibilities. Developmentally, either the surrounding granular sarcoplasm or the muscle nucleus could come first. Since muscle nuclei are capable of processing proteins important in granular sarcoplasm structure, including receptor proteins and structural proteins responsible for the adhesion of receptors into clusters (Gordon et al., 1992; Moscoso et al., 1994), it seems more likely that the nuclei are responsible for manufacturing the granular sarcoplasm seen around them. The positioning of the granular sarcoplasm surrounding the nuclei further hints to the nuclei being the focus around which sarcoplasm becomes aggregated rather than the other way
around. Defined connections appear to link the myonuclei with the surrounding granular sarcoplasm. Whether these are conduits by which newly synthesised sarcoplasm is leaving the nucleus or merely adhesion sites is unknown. In established muscle systems, myonuclei were found to be preferentially located adjacent to nerve terminals (Harrington and Atwood, 1995). As a result, it is likely that these are possible acceptance sites on the muscle open to innervation. These “acceptance pads” could be triggered by the muscle in response to denervation, through a signal released from nearby regenerating axons or a combination of both.

Upon denervation, a number of events may occur. The myonuclei associated with the former synaptic sites may become mobile. In vertebrates it’s been observed that myonuclei associated with ACh receptors tend to be stationary, while those without become more transitory in nature (Englander and Rubin, 1987). If nuclei do become more transitory following terminal degradation, they could be attracted by regenerating axons growing close to the muscle surface and then subsequently affected by neuronal signals that initiate granular sarcoplasm production. All signals, whether it simply be neurotransmitter or a more specialized agrin-like molecule, must be diffusible as these structures are observed in the absence of neuronal contact. It is possible that the regenerating nerve may first attract glutamate receptors which in turn attract the myonucleus, immobilizing it on contact.

Conversely, myonuclei may be recycled and become attracted to former synaptic sites following denervation. They may become anchored to areas along the former path of innervation and in turn attract regenerating axons. The surrounding granular sarcoplasm may be precursory in nature, triggered by the denervated muscle and perhaps instrumental in attracting the axon or it could be triggered by a regenerating axon following its attraction of the nucleus. Since the onset of receptor aggregation is dependent on the presence of
motoneurons (Broadie and Bate, 1993; Hall and Sanes, 1993; Currie et al, 1995), the regenerating nerve must be present for mature granular sarcoplasm to develop. Stationary nuclei situated near former innervation networks would explain why regenerating nerves are often seen to mimic former innervation maps (Krause and Vélez, 1995). However, the remnants of former nerves could also emit a signal that attracts the regenerating nerve resulting in the same trend. Thus both scenarios of the nuclei attracting the nerve and the nerve attracting the nuclei are equally plausible.

The one significant influence the muscle seems to exert is in defining a limited acceptance to the number of regenerating neurons. In all the muscle fibres that were electrophysiologically measured, no fibre indicated the acceptance of more than three axons. This is a little below the number of axons it accepts in its natural state (Kennedy and Takeda, 1965b) and implies the existence of a lowered carrying capacity. Carrying capacities of muscles have been implied in terms of the total number of synaptic connections that can be made. The quest to induce hyperinnervation in most systems has proven to be difficult (Grinnell, 1976; Jacobson, 1978). The study on the developmental muscle mutations in Drosophila found that while connections were possible on the least afflicted mbc mutant, extrasynaptic active zones occurred 55% of the time (Prokop et al., 1996). It was proffered that these ectopic active zones were due to the decreased surface area of the muscle as a result of the mutation. While the nerve was not affected and maintained its autonomous programme in assembling active zones, the accommodating capability of the muscle decreased in concordance to its decreased size. We also observed a much greater degree of ectopic active zones in our regenerated neurons. Perhaps the transplanted nerves naturally innervate a muscle fibre with a greater surface area and consequently assembled more active zones than are required for this particular host muscle. This limitation by the muscle may be due to a fixed number of the myonuclei discussed.
above. If these myonuclei are responsible for differentiation of the postsynaptic membrane, as they evidently appear to be, a limit on their number would effectively limit the number of connections that would be allowed. Thus, while the muscle plays an integral role in restricting the quantity of innervation, it seems to have little control over the quality of innervation. Muscular presence is necessary for the alignment and consolidation of active zones presumably triggered by a neuronal signal and resulting in an involved series of interactions between the nerve and muscle.
SUMMARY

The objective of this study was to gain a better understanding of the relative involvement of the motoneuron and its target in determining synaptic structure and function during regeneration. To this end a neuromuscular system was chosen that had previously been found to accept allotransplanted nerves from other animals within the same species. The denervated tonic superficial muscle system of Procambarus clarkii not only accepts an allotransplant of its contemporary motoneuron (Krause and Velez, 1995), but from completely foreign neurons as well (Krause et al., 1998). This provided an opportunity to introduce a mixed nerve containing a considerable number of both tonic and phasic motoneurons to a normally tonic system. As a result the third root of the sixth ganglion was extracted from a donor animal and transplanted onto the denervated SFM of a recipient. The mere ability of foreign innervation to successfully regenerate, along with a comparison of electrophysiological and structural properties of the resulting novel synapses to normal parameters, will provide insight into the mechanisms involved in synaptogenesis.

Electrophysiology showed that viable synaptic connections were made on the denervated SFM. EPSPs evoked by stimulating the nerve found on the muscle not only revealed synaptic efficacies similar to that seen in native tonic innervation, but in addition EPSPs with far greater amplitudes and less facilitative ability were also found. Such EPSP properties are more indicative of phasic innervation. Structural analysis substantiated these findings. Populations of synapses much greater in area with significantly more complex dense bars per synapse as well as smaller than normal synapses with considerably longer dense bars were found in conjunction to populations that parallel properties found in control conditions. These structural anomalies are probably responsible for the more phasic-like EPSPs recorded (King et al., 1996). Thus, it tentatively appears both tonic and phasic motoneurons from the allotransplanted mixed nerve were successful in innervating the tonic
target muscle.

The ability of foreign nerves to successfully innervate muscles indicates that common properties to both the presynaptic and postsynaptic membrane must be involved in synaptogenesis. The competence of the regenerating nerve in sprouting out to the target indicates the presence of a diffusible signal originating from the muscle informing the nerve of its denervated state. Again this signal must be common in nature, affecting motoneurons alike.

The varying expression of synaptic phenotypes suggests that an autonomous programme inherent to the motoneuron is responsible for deciding synaptic structure and function. In cases where retrograde influences are apparent (Atwood and Bittner, 1971; Davis and Murphey, 1993; Katz et al., 1993), a property specific to the post-synaptic target is probably responsible with the capability of affecting synaptic properties within a genetically defined range dictated by the neuron. My study did not examine variation within a single motoneuron but rather variation between electrophysiological and structural properties in general and in this instance the SFM fibres did not appear to exert a retrograde influence.

The muscle is accommodating, accepting both tonic and phasic innervation. Favouritism, or lack there of, for the acceptance of one type of motoneuron over the other could not be assessed. The presence of myonuclei with surrounding granular sarcoplasm lacking innervation is a peculiarity of crustacean muscle regenerating innervation. Consequently, it is probably representative of a stage in nerve terminal differentiation and synaptogenesis, most likely serving as some sort of an “acceptance pad” to outgrowing motoneurons. The muscle appears to have little effect on the determination of synaptic properties, accepting whatever appears to come first. However, it does seem to set a limit to the number of
motoneurons it will accept, as in all of the electrophysiological recordings no more than three motoneurons appear to innervate a single muscle fibre. This carrying capacity of three motoneurons is a little below the level of innervation found under normal conditions (Kennedy and Takeda, 1965b).

The absence of spontaneous activity elicited by the regenerating nerve prior to stimulation suggests that activity is not a prime factor for shaping synaptic properties as determined by the motoneuronal autonomous programme. Likewise, little activity appears to be necessary for the consolidation of synapses. This independence from activity may be a property of adult systems.
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