Presynaptic and Postsynaptic Competition in Models for the Development of Neuromuscular Connections

by Carl E Rasmussen[1] & David J Willshaw

Centre for Cognitive Science

University of Edinburgh

2 Buccleuch Place

Edinburgh EH8 9LW

Scotland

E-mail: *carl@cns.ed.ac.uk & david@cns.ed.ac.uk*

Abstract

The development of the nervous system involves in many cases interactions on a local scale rather than the execution of a fully specified genetic blueprint. The problem is to discover the nature of these interactions and the factors on which they depend.

The withdrawal of polyinnervation in developing muscle is an example where such competitive interactions play an important role. We examine the possible types of competition in formal

[1]Present address: Department of Biophysics, Technical University of Denmark, Building 307, DK–2800 Lyngby, Denmark

models that have plausible biological implementations. By relating the behaviour of the models to the anatomical and physiological findings we show that a model that incorporates two types of competition is superior to others. Analysis suggests that the phenomenon of intrinsic withdrawal is a side effect of the competitive mechanisms rather than a separate non–competitive feature. Full scale computer simulations have been used to confirm the capabilities of this model.

1 Introduction

In several parts of the vertebrate nervous system the development of nerve connections involves an initial stage of superinnervation followed by withdrawal of axon terminals until just one contact per target cell remains (Redfern, 1970; Jansen & Fladby, 1990).

The best studied case is the innervation of mammalian skeletal muscle by its motor nerve. During prenatal development, the axons of the motor neurons grow towards their target muscle. Near the muscle each axon arborizes to innervate a large number of muscle fibres. At birth each muscle fibre is contacted by terminals from a small number of motor neurons. During the first few weeks after birth, axons lose some of their terminals until each muscle endplate is innervated by a single terminal (fig 1). A similar pattern of events is found during reinnervation of adult muscle after injury to the motor nerve (McArdle, 1975).

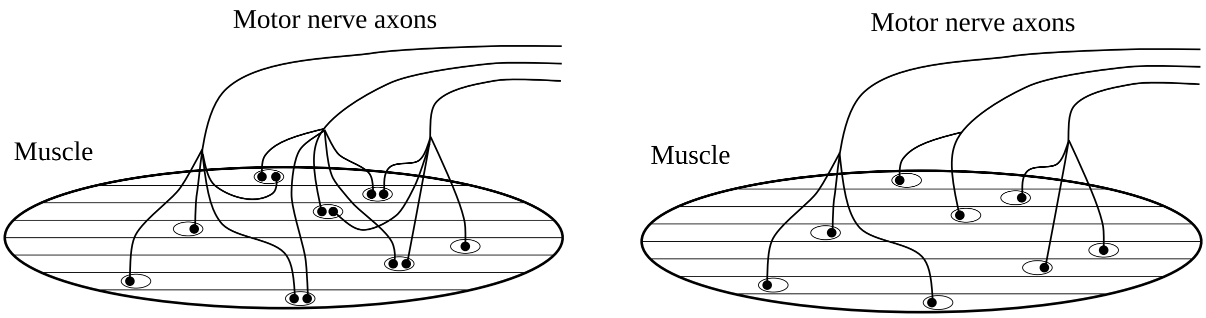


Figure 1: The muscle is innervated by a motor nerve containing 3 axons. Each muscle fibre has a single endplate. (a) Early stage. Several fibres are contacted by more than one terminal. (b) Later stage. Polyinnervation has been withdrawn, leaving all endplates with a single terminal.

The neuromuscular system has been the subject of a large number of experiments which have given clues as to the nature of the controlling mechanisms. However, these mechanisms are still largely unknown. The loss of the extra terminals innervating the muscle is not due to neuron death or addition of muscle fibres, since the numbers of neurons and fibres do not change significantly during the period in question. Microscopy studies show that terminals are actually resorbed into the motor neuron.

Several attempts have been made to construct formal models for the withdrawal of superinnervation during the development of neuromuscular connections. Since the actual biochemical reactions that control the development of the pattern of neuromuscular innervation are unknown, assumptions in the different models must be largely justified by their ability to reproduce the key experimental findings.

The main concern has been to formulate models that can achieve single innervation. At this level, the problem is that very different ideas can generate the same qualitative behaviour. In order to reduce the number of possible mechanisms, it is important to design models that are applicable to a wider range of experimental findings. It is equally important to consider whether the models are biologically feasible in terms of the physical and chemical reactions they require.

In this paper we examine the basic ideas that have been proposed for the development of neuromuscular connections and we then investigate how far these ideas can be extended. Firstly, we review briefly the anatomical and physiological findings concerning the withdrawal of superinnervation. In section 3 we discuss existing models employing competitive mechanisms. In section 4 a more recent model that combines two types of competition is described. Section 5 discusses how the implementation of this model can be improved and reports the stability analysis that we performed on our own version of this model. In section 6 we present computer simulations illustrating various properties of the model.

2 Experimental Findings

In mouse, rat, rabbit and cat, there is an initial phase of polyinnervation of muscle fibres which develops into a state of single innervation (Redfern, 1970; Jansen & Fladby, 1990). The same pattern of events occurs during the reinnervation of adult muscle following transection of the nerve (McArdle, 1975) and when foreign nerves are transplanted to denervated muscles (Taxt, 1983).

2.1 Normal development

Around the time of birth all muscles are multiply innervated, leading to complete single innervation a few days later. In any one muscle the amount of initial innervation ranges from fibre to fibre with very few, if any, uninnervated muscle fibres.

The average amount of initial innervation (the mean number of terminals per endplate) varies from 2.7 in the rat lumbrical muscle (Betz *et al.*, 1979) to 6.0 in the mouse soleus (Fladby & Jansen, 1988). In the rabbit diaphragm muscle, singly innervated muscle fibres first appear 5 days prenatally, and single innervation is complete 7 days postnatally (Bixby & Van Essen, 1979). In rabbit soleus this interval stretches from 3 to 11 days postnatally (Bixby & Van Essen, 1979) and in rat soleus from 9 to 15 days postnatally (Brown *et al.*, 1976). Since in the initial stages terminals can be withdrawn without causing any single innervation, the withdrawal process may start a few days earlier than the onset of single innervation.

The extent of innervation can also be expressed in terms of the decrease in *motor unit size* (the number of fibres contacted by a given motor axon). The variability in size amongst the motor units of the rat soleus muscle decreases substantially during the elimination of polyinnervation. Brown *et al.* (1976) found that an initial 8–fold spread (defined as the ratio of the size of the largest motor unit to the smallest) was reduced to a 3–fold spread in the mature muscle. In contrast, no such reduction was seen in the rabbit soleus muscle (Gordon & Van Essen, 1981).

2.2 Partial denervation experiments

In both neonates and adults, muscles can be partially denervated by removing some of the axons in the motor nerve. This experimental paradigm has been used to investigate the role of various possible mechanisms in the withdrawal of superinnervation.

It has been shown that after neonatal partial denervation, the average size of the remaining motor units developed is larger than normal (Thompson & Jansen, 1977; Fladby & Jansen, 1987). This demonstrates that the fate of a terminal depends on the presence of other terminals, which leads to the idea of a competitive mechanism: terminals from different axons compete for the endplate.

However, this kind of competitive mechanism alone cannot account for all findings. Partial denervation experiments where all but a single motor axon were removed were used to investigate whether terminals at singly innervated endplates could be withdrawn, even in the absence of competition from other terminals. One set of experiments on neonatal rat lumbrical muscle (Betz *et al.*, 1980) indicated no withdrawal under these conditions. On the contrary, in experiments involving partial denervation of the mouse soleus, Fladby & Jansen (1987) calculated that the number of innervated muscle fibres in the adult was less than the number innervated initially; some terminals must have withdrawn to leave uninnervated endplates. This has led some authors to suggest the mechanism of *intrinsic withdrawal*, by which a certain small number of the initial set of connections made by each motor axon always withdraw (Thompson & Jansen, 1977; Fladby & Jansen, 1987).

2.3 Sprouting and reinnervation

In the adult, the existence of uninnervated fibres provokes *sprouting*: new terminals grow from the terminal processes of existing axons to innervate uninnervated fibres nearby.

If a large enough number of axons are left intact after partial denervation in the adult, the extent of sprouting from these axons will be enough to cover the entire muscle. This is shown by experiments on the rat lumbrical muscle which is innervated by the sural nerve and the lateral plantar nerve. Following damage to the sural nerve, the lateral plantar nerve expands its territory to innervate the entire muscle. On subsequent reinnervation, the sural nerve can reoccupy some of the muscle fibres that became occupied by the intact nerve (Ribchester & Taxt, 1983). The regrowing nerve innervates the endplates that already receive connections from the undamaged axons, and is successful in competing with the intact axons, which gradually retreat. Ribchester (1988) showed that muscles left with only very few axons were not completely innervated, indicating that sprouting only takes place to a limited extent.

2.4 Additional effects

The final pattern of connections is related to a number of other factors including

Activity dependence The level of activity in both nerve and muscle plays an important role in the elimination of terminals. Inactivity, by means of pharmacological block, prevents withdrawal of terminals, and induces sprouting (Thompson, 1985; Brown *et al.*, 1982). Increased activity induced by electrical stimulation of the nerve has the opposite effect of accelerating withdrawal (O'Brian *et al.*, 1978; Thompson, 1983). There are also selective effects. When blocked and unblocked motor neurons reinnervate the same muscle, in some cases the unblocked motor units are found to be larger than the blocked ones (Ribchester & Taxt, 1983); in some cases they are smaller (Callaway *et al.*, 1989).

Fibre and muscle types There are different types of both motor neurons and muscle fibres, classified according to their preferred frequency of activity.

In rabbit soleus (Gordon & Van Essen, 1985) and mouse soleus (Fladby & Jansen, 1988), motor units are clearly differentiated into fast and slow types even during the initial stage of polyinnervation. Thus each motor neuron already innervates a fairly homogeneous group of fibres.

Rat soleus contains predominantly slow muscle fibres (Close, 1967) whereas the rabbit soleus is more inhomogeneous, consisting of $\frac{1}{3}$ slow fibres and $\frac{2}{3}$ fast fibres (Jansen & Fladby, 1990) and an approximately equal number of slow and fast neurons (Gordon & Van Essen, 1985). This means that in rabbit soleus the average size of fast motor units must be twice that of the slow motor units.

Topological maps Regional localisation of connections may be involved in determining the pattern of innervation. There is some evidence that the distribution of endplates in the muscle innervated by a given motor neuron reflects the position of that motor neuron in the spinal cord (Laskowski & Sanes, 1988).

In this paper we examine the basic mechanism of competition whereby the number and distribution of contacts made by each motor neuron drives the withdrawal of superinnervation. We do not consider the effects of activity, type matching and topographical arrangement at this stage.

3 Mechanisms & models

The fact that the final pattern of connections can be altered shows that a mechanism according to which certain terminals are preprogrammed to withdraw cannot on its own account for the experimental findings. The fate of a terminal depends on other terminals; ie, the mechanism for withdrawal involves competition between terminals.

Models invoking competitive mechanisms are well known to theoretical neuroscience. The most common types are those involving competition between the various terminals of each presynaptic neuron (Willshaw & von der Malsburg, 1979) or between the terminals connecting each postsynaptic neuron (von der Malsburg, 1973). In most cases, the action of such mechanisms have been expressed as the conservation of some measure of the total amount of synaptic strength available to the neuron on question. How such a conservation process is implemented is not usually discussed.

In the development of the neuromuscular junction the presynaptic elements (motor neurons) compete for control of the postsynaptic element (endplate). Thus there must be some kind of competition at the postsynaptic site.

3.1 A model relying on competition for a postsynaptic resource

The idea behind the model proposed by Gouzé *et al.* (1983) is that the terminals at each endplate compete for a share of a certain substance, assumed to be available in a limited amount at each endplate. This substance is gradually transferred to the terminals at that endplate, where it is converted into *survival factor*. The rate of transfer to a particular terminal is assumed to be a steeply rising function of the amount of postsynaptic substance already taken up by the terminal. The small random differences in the initial amount of factor assigned to terminals become magnified by this process until just one terminal survives.

According to this model, the elimination of terminals at each endplate occurs independently of the elimination at other endplates. All motor neurons are therefore regarded as equivalent and there is no way by which terminals from a particular motor neuron can affect its other terminals. This would be required in order to explain why large motor units reduce in size more than smaller ones, and how one nerve can regain territory at the expense of another one. Although a model relying on the mechanism of competition for a postsynaptic resource alone can account for the basic phenomenon, it cannot account for a substantial number of related experimental findings.

3.2 Importance of a limited presynaptic capacity

Many of the experimental results indicate that motor neurons each have the same, limited capacity to maintain terminals.

1. **Limited number of terminals per axon**

    - Perhaps the clearest indication of this is the result of experiments of partial denervation on the neonatal mouse soleus (Fladby & Jansen, 1987). Removal of most of the motor units leads to incomplete innervation in the adult muscle. Under these conditions the average motor unit size is found to be independent of the remaining number of motor units. This suggests that each neuron can maintain only a limited number of terminals.
    - This notion is further supported by the finding that in reinnervation experiments motor neurons have only a limited ability to sprout.

2. **Larger motor units are less competitive**

    If presynaptic capacity were limited, the terminals of larger motor units would be weaker and therefore less competitive than those of smaller motor units.

    - This would account for the reduction in spread of motor unit sizes during withdrawal in rat soleus.
    - It would also be consistent with the finding that during reinnervation by the severed nerve of partially denervated muscle, the regenerating axons are able to take over control of some of the endplates from the intact neurons that had expanded their territory.

    Gordon & van Essen (1981) found no reduction in the variability of motor unit sizes in the rabbit soleus. However, it should be noted that rabbit soleus is a very inhomogeneous muscle, whereas rat soleus contains predominantly slow fibres. If motor neurons innervate almost exclusively fibres of their own type (slow or fast), there may be separate reductions in the spread of motor unit sizes within the slow and fast parts of the muscle, without an overall reduction being seen.

Perhaps the most straightforward way of expressing a presynaptic sum rule is to assume that each neuron possesses a fixed *amount* of a certain factor, which is essential for the maintenance of terminals (Willshaw, 1981). A second way is to assume a fixed *rate* of production of factor, with a limited halflife. This would correspond to a metabolic turnover of substance for each neuron. There is no direct experimental evidence as to which of these two implementations (or any other) is the most likely.

3.3 Model using competition for a presynaptic resource

Willshaw's earlier model (1981) was intended to represent the idea of O'Brien *et al.* (1978), who proposed that terminals release a digestive enzyme that has a degrading effect on all terminals at an endplate. In this model there are two factors that influence growth:

1. The survival strength of a terminal increases at a rate proportional to its existing strength.
2. Survival strengths are weakened according to the total amount of survival strength available at its endplate. This expresses the effect of the digestive enzyme.

In this model a *presynaptic conservation rule* is employed, which expresses the intuition that the total amount of presynaptic resource is limited (Lichtman, 1977). The positive and negative changes applied to the survival strengths are scaled so that the total survival strength assigned to the terminals of each motor neuron stays at a fixed level.

A system functioning according to these rules will develop from an initial state of polyinnervation to a state where all fibres receive single innervation. This model also provides an explanation for the decrease in the range of motor unit size during development. Since the terminals of the larger motor units will be weaker and therefore less competitive than terminals from smaller units, the larger units will lose comparatively more terminals.

However, conservation of survival strength was introduced into this model as a mathematical constraint without specifying the biological processes that would implement it. This is a problem since the interactions producing growth and decay probably originate in very different biochemical processes (ie terminal growth and enzymatic digestion). These two factors must be finely balanced in order to ensure presynaptic conservation and ultimately the stability of the system. It is hard to see how the necessary coupling between these two very different mechanisms could be achieved.

One way around the problem of implementing a presynaptic sumrule is to assume that there is competition for a presynaptic *substance*, rather than using an abstract mathematical rule. The amount of presynaptic substance will be limited thus ensuring that the presynaptic sumrule is implemented in a biologically plausible way.

3.4 Combined presynaptic and postsynaptic conservation

Both presynaptic and postsynaptic competition have desirable effects. Postsynaptic competition is necessary to achieve single innervation and presynaptic competition gives an account of many experimental findings. There are problems with Willshaw's earlier model due to the lack of an actual substance at the presynaptic site.

This leads naturally to the idea of combining the two types of competition for substance in a single model, as proposed by Bennett & Robinson (1989). We call this type of model a *dual constraint model*, or DCM.

4 Bennett & Robinson's Dual Constraint Model

This model (Bennett & Robinson, 1989) combines mechanisms of competition for a presynaptic substance with competition for a postsynaptic substance. In the next two sections we show how we have developed this model.

We first describe the model, using our own notation. There are N motor neurons and M muscle fibres. A particular motor neuron is indexed by n, a muscle fibre by m and a terminal by nm. The key dependent variables are the quantities A_n, A_{nm}, B_m and C_{nm}, defined below, which are all functions of time. Since not all neurons have terminals at all muscle fibres, the sums taken over neurons and muscle fibres are only over the terms for which terminals exist.

4.1 Description of the model

In this model a reversible reaction between the presynaptic substance A and postsynaptic substance B produces binding complexes C. These are essential to the maintenance of terminals, the size of terminal nm assumed to be directly proportional to C_{nm}.

The reactions take place in the synaptic cleft, where presynaptic molecules are found in the terminal membranes and postsynaptic molecules are located in the endplate membrane. The reversible chemical reaction takes one molecule of each of A and B to produce one of C

$$A_{nm} + B_m \rightleftharpoons C_{nm} \tag{1}$$

Each motor neuron n has a fixed amount A_0 of presynaptic substance A available to it. Molecules of A can be located either in the cell soma, in amount A_n, or in one of the terminals of the neuron. In terminal nm, A can either be unbound in the terminal membrane, in amount A_{nm}, or bound, in amount C_{nm}. The conservation equation for A is

$$A_0 = A_n + \sum_{j=1}^{M} A_{nj} + \sum_{j=1}^{M} C_{nj} \tag{2}$$

Bennett & Robinson assumed that the amount of unbound presynaptic substance in a terminal is proportional to (i) the size of the terminal C_{nm} and (ii) the amount of presynaptic substance in the cell soma A_n, yielding

$$A_{nm} = K C_{nm} A_n \tag{3}$$

where K is a constant.

Each endplate m has a fixed amount B_0 of postsynaptic substance B available to it. Molecules of B can either be unbound in the endplate membrane, in amount B_m, or bound, in amount C_{nm}, at the site. The conservation equation for B is

$$B_0 = B_m + \sum_{i=1}^{N} C_{im} \tag{4}$$

4.2 Reaction kinetics

The reversible chemical reaction between A_{nm} and B_m involves a forward and a backward reaction. The forward reaction rate is taken to be proportional to the product of the amounts of the pre-synaptic substance A_{nm}, the postsynaptic substance B_m and a fixed power μ of the amount of binding complex C_{nm} that are locally available. The backward reaction rate is taken to be proportional to the amount of binding complex, C_{nm}.

$$\frac{dC_{nm}}{dt} = K_1 A_{nm} B_m C_{nm}^{\mu} - K_2 C_{nm} \tag{5}$$

where K_1 and K_2 are rate constants. The justification for including a term involving C_{nm}^{μ} is that electrical activity in the endplate region could produce electromigration of molecules of B in the endplate membrane. The result is that the larger terminals will have an ability to attract the postsynaptic molecules to the endplate region immediately under the terminal – thus favouring the forward reaction rate at these terminals. This is implemented by fixing μ at a small positive value.

We use (2) – (4) to express B_m and A_{nm} in terms of C_{nm}

$$A_{nm} = K \frac{A_0 - \sum_{j=1}^{M} C_{nj}}{1 + K \sum_{j=1}^{M} C_{nj}} C_{nm} \quad \text{and} \quad B_m = B_0 - \sum_{i=1}^{N} C_{im} \tag{6}$$

Introducing these expressions for A_{nm} and B_m into (5) gives a set of first-order differential equations for how C_{nm} changes over time.

4.3 Simulation results

Bennett & Robinson chose the value of the ratio A_0/B_0 so as to make the total amount of presynaptic substance equal to the total amount of postsynaptic substance; ie, $A_0/B_0 = M/N$.

In their numerical calculations, a set of terminals was chosen at random and each was given the same small amount of the binding complex C. Numerical solutions of the set of differential equations for C_{nm} as defined by (5) & (6) demonstrated that during the initial phase all terminals grew, but eventually all but a single terminal at each endplate retracted. In most cases, a state of single innervation was reached.

5 Investigation of Dual Constraint models

In this section we address in detail several issues of implementation of DCM. We then derive the conditions for stability of the state of single innervation. This led us to a novel interpretation of several experimental phenomena.

5.1 Implementation

5.1.1 Transport of A

Bennett & Robinson do not discuss what processes could give rise to the distribution of A given by (3). We suggest a scheme of transport mechanisms that should be easy to implement.

A terminal that is growing rapidly must have a net flow of A *to* the terminal, whereas a terminal that is shrinking will have a net flow *from* the terminal; both anterograde and retrograde transport is required. We use the familiar physical principle that the rate of transport is proportional to the concentration of substance at the sending site.

The rate of *anterograde* transport down the axon of the motor neuron is assumed to be proportional to the concentration of presynaptic substance in the cell body. Since the size of the cell body is constant, this concentration is proportional to the number of presynaptic molecules there, A_n. We suggest the simplifying approximation that when the molecules reach the arborisation of the axon, this anterograde flow becomes divided evenly between the ν_n terminals of the neuron. This will probably require that all the terminal sprouts arborize from the same point and have similar characteristics. This division of flow should be simple to implement since it does not require a sophisticated computation - at the point of arborisation no knowledge is needed about the size of the terminal at the end of each axon branch relative to the number and size of the other terminals.

The rate of *retrograde* transport from each terminal is also taken to be proportional to the concentration of presynaptic molecules. In the two-dimensional terminal membrane, this concentration is proportional to A_{nm}/C_{nm}, given that the size (area) of a terminal is proportional to C_{nm}. This equation could give rise to difficulties if C_{nm} were allowed have values close to zero. This situation will never arise however since very small terminals are removed explicitly (see below). The transport rates are therefore

$$T_{anterograde} = \gamma \frac{A_n}{\nu_n} \qquad T_{retrograde} = \delta \frac{A_{nm}}{C_{nm}} \tag{7}$$

where γ and δ are rate constants. These two equations combine to form a differential equation for the transport of A

$$\frac{dA_{nm}}{dt} = \gamma \frac{A_n}{\nu_n} - \delta \frac{A_{nm}}{C_{nm}} \tag{8}$$

Equilibrium is achieved when

$$\frac{dA_{nm}}{dt} = 0 \Longleftrightarrow A_{nm} = \frac{\gamma}{\delta \nu_n} A_n C_{nm} \tag{9}$$

If the transport rate constants (γ and δ) are large, the system will settle quickly towards equilibrium. During withdrawal the equilibrium point changes continuously (since it depends on $A_n C_{nm}$), and so large values of γ and δ will cause the system to be close to equilibrium at all times.

By equating the constant K of (3) used in the original model of Bennett & Robinson with $\gamma/\delta\nu_n$ of (9), it can be seen that Bennett & Robinson's equation (3) corresponds to the steady state in our novel interpretation. An important difference is that the new term has a dependency on ν_n.

5.1.2 Explicit withdrawal of small terminals

Terminals that possess a very small amount of C_{nm} are considered by Bennett & Robinson to be eliminated. This elimination does not have any particular consequence for their equations.

However, in our scheme the substance A must be transported only to the terminals that do exist, and so explicit withdrawal of terminals is required. We regard any terminal for which the value of C_{nm} has fallen below a small threshold value θ as being withdrawn. This is done by removing the equations for terminal nm and decreasing the corresponding ν_n by one. The tiny amounts of A and B held by the terminal are resorbed into the motor neuron and the muscle endplate respectively.

The model proposed by Willshaw (1981) has been criticized (Gouzé *et al.*, 1983) for the discontinuity introduced when a terminal withdraws. In our model a similar discontinuity will arise (stepwise decrement of ν_n), but here it is supported by the underlying mechanisms.

5.1.3 Clarification of justification for reaction rates

The account of the chemical reaction rates given by Bennett & Robinson does not justify very clearly the relation between terminal size and reaction rate. We offer the following interpretation.

Molecules of A and B in the pre- and postsynaptic membranes move freely about in the membranes. The rate of the forward reaction is taken to be proportional to the probability that a molecule of A and one of B collide. This probability is proportional to the product of the concentrations of A and B and the size of the reaction surface (the terminal membrane area) which is proportional to C. The backward reaction rate is thought to be proportional to the amount C_{nm} of binding complexes that are available to break up. This would give

$$\frac{dC_{nm}}{dt} = \alpha\frac{A_{nm}}{C_{nm}}B_mC_{nm} - \beta C_{nm} = \alpha A_{nm}B_m - \beta C_{nm} \tag{10}$$

In order to achieve single innervation, however, it is important to favour larger terminals over smaller ones. According to (5), the forward reaction rate has a dependency on C_{nm}. We introduce a linear dependency, to give

$$\frac{dC_{nm}}{dt} = \alpha A_{nm}B_mC_{nm} - \beta C_{nm} \tag{11}$$

We find it difficult to justify inclusion of this dependency on C_{nm} beyond saying that a way of favouring larger terminals (ie terminals possess a large amount of C_{nm}) over smaller ones is needed to achieve single innervation. It is not clear to us what the physical mechanisms underlying this dependency could be. Bennett & Robinson proposed electromigration, whereby large terminals attract postsynaptic molecules by the electrical effects of activity. Larger terminals could release larger quantities of neurotransmitter, therefore causing a larger electric field and thus attracting more molecules than smaller terminals.

5.2 Mathematical Analysis

The development of connections according to the revised model of Bennett and Robinson is specified by two sets of first-order differential equations. Using equations (2), (4), (8), (11), for terminal nm these are

$$\frac{dC_{nm}}{dt} = \alpha A_{nm}(B_0 - \sum_{i=1}^{N} C_{im})C_{nm} - \beta C_{nm} \tag{12}$$

$$\frac{dA_{nm}}{dt} = \frac{\gamma}{\nu_n}(A_0 - \sum_{j=1}^{M} A_{nj} - \sum_{j=1}^{M} C_{nj}) - \delta\frac{A_{nm}}{C_{nm}} \tag{13}$$

These equations are difficult to handle analytically. Because of the nonlinearity of the equations we have not been able to solve them in the general case.

Our computer simulations (section 6) suggest that states of polyinnervation are unstable, but we have not been able to prove this analytically. Analysis of the single innervation case is however possible.

5.2.1 Stability of the single innervation state

We will now derive the conditions under which a state of single innervation is a stable state, using perturbation analysis. We test whether a given equilibrium state is stable against noise (with no DC component) in the values of C_{nm} and A_{nm}.

Let $\tilde{A}_{nm}$ and $\tilde{C}_{nm}$ denote an equilibrium state. Further let ϕ_{nm} and ψ_{nm} denote small perturbations to an equilibrium state given by

$$\frac{d\tilde{C}_{nm}}{dt} = 0 \qquad C_{nm} = \tilde{C}_{nm} + \phi_{nm} \tag{14}$$

$$\frac{d\tilde{A}_{nm}}{dt} = 0 \qquad A_{nm} = \tilde{A}_{nm} + \psi_{nm} \tag{15}$$

The first order (linear) Taylor expansion about the equilibrium point is given by

$$\begin{aligned}\frac{d\phi_{nm}}{dt} &= \alpha(\tilde{A}_{nm} + \psi_{nm})[B_0 - \sum_{i=1}^{N} \tilde{C}_{im} - \sum_{i=1}^{N} \phi_{im}](\tilde{C}_{nm} + \phi_{nm}) - \beta(\tilde{C}_{nm} + \phi_{nm}) \\ &\simeq \alpha\tilde{C}_{nm}\left([B_0 - \sum_{i=1}^{N} \tilde{C}_{im}]\psi_{nm} - \tilde{A}_{nm}\sum_{i=1}^{N} \phi_{im}\right) \end{aligned} \tag{16}$$

$$
\begin{aligned}
\frac{d\psi_{nm}}{dt} &= \frac{\gamma}{\nu_n}[A_0 - \sum_{j=1}^{M}\tilde{A}_{nj} - \sum_{j=1}^{M}\psi_{nj} - \sum_{j=1}^{M}\tilde{C}_{nj} - \sum_{j=1}^{M}\phi_{nj}] - \delta\frac{\tilde{A}_{nm}+\psi_{nm}}{\tilde{C}_{nm}+\phi_{nm}} \\
&\simeq \frac{\delta}{\tilde{C}_{nm}^2}(\tilde{A}_{nm}\phi_{nm} - \tilde{C}_{nm}\psi_{nm}) - \frac{\gamma}{\nu_n}\left[\sum_{j=1}^{M}\phi_{nj} + \sum_{j=1}^{M}\psi_{nj}\right] \qquad (17)
\end{aligned}
$$

Writing this in matrix form by lining up all ϕ_{nm}'s and ψ_{nm}'s to form a vector $\mathbf{x}$, we have

$$
\frac{d\mathbf{x}}{dt} = \mathcal{W}\mathbf{x} \quad \text{with solutions} \quad \mathbf{x} = \sum_i k_i \mathbf{u}_i \exp(\lambda_i t) \qquad (18)
$$

where k_i are constants, $\mathbf{u}_i$ are eigenvectors of $\mathcal{W}$ and λ_i the corresponding eigenvalues. This shows that upon perturbation the system will settle back towards the equilibrium point $\mathbf{x}=\mathbf{0}$ if all $\Re(\lambda_i) < 0$ (where $\Re$ denotes the real part). Thus, the equilibrium point corresponds to a stable point if all eigenvalues λ_i have negative real parts.

It is important to note that in the single innervation case all motor units are completely independent. Therefore, let us look at a single motor unit. For any one of its terminals nm in the single innervation state we have $\sum_{i=1}^{N} C_{im} = C_{nm}$. Introducing this in (12) and solving (12) & (13) for $\tilde{C}_{nm}$ and $\tilde{A}_{nm}$ we deduce that all $\tilde{C}_{nm}$ and all $\tilde{A}_{nm}$ belonging to the same motor unit will take the same value – called $\tilde{C}$ and $\tilde{A}$ respectively. We now make a small scale version of vector $\mathbf{x}$, by including only ϕ's and ψ's corresponding to actual terminals of the neuron. The neuron in question has ν terminals, so

$$
\mathbf{x} = (\phi_1, \phi_2, \ldots, \phi_\nu, \psi_1, \psi_2, \ldots, \psi_\nu)^T \qquad (19)
$$

The eigenvalues λ_i of the corresponding matrix $\mathcal{W}$ are solutions of $\det(\mathcal{W} - \lambda\mathcal{I}) = 0$ where $\mathcal{I}$ is the unit matrix of size 2ν. The general form of this equation is

$$
\begin{vmatrix}
a-\lambda & 0 & \cdots & 0 & 0 & b & 0 & \cdots & 0 & 0 \\
0 & a-\lambda & \cdots & 0 & 0 & 0 & b & \cdots & 0 & 0 \\
\vdots & \vdots & & \vdots & \vdots & \vdots & \vdots & & \vdots & \vdots \\
0 & 0 & \cdots & a-\lambda & 0 & 0 & 0 & \cdots & b & 0 \\
0 & 0 & \cdots & 0 & a-\lambda & 0 & 0 & \cdots & 0 & b \\
c+f & f & \cdots & f & f & g-\lambda & f & \cdots & f & f \\
f & c+f & \cdots & f & f & f & g-\lambda & \cdots & f & f \\
\vdots & \vdots & & \vdots & \vdots & \vdots & \vdots & & \vdots & \vdots \\
f & f & \cdots & c+f & f & f & f & \cdots & g-\lambda & f \\
f & f & \cdots & f & c+f & f & f & \cdots & f & g-\lambda
\end{vmatrix} = 0 \qquad (20)
$$

where

$$
a = -\alpha\tilde{C}\tilde{A} \quad b = \alpha\tilde{C}[B_0 - \tilde{C}] \quad c = \frac{\delta\tilde{A}}{\tilde{C}^2} \quad d = -\frac{\delta}{\tilde{C}} \quad f = -\frac{\gamma}{\nu} \quad g = d + f \qquad (21)
$$

The matrix equation (20) can by the following operations

$$\begin{aligned}
\text{for } i = 1\ldots\nu : \mathrm{row}_{\nu+i} &:= \mathrm{row}_{\nu+i} - \frac{d-\lambda}{b}\mathrm{row}_i - \frac{f}{b}\sum_{j=1}^{\nu}\mathrm{row}_j \\
\text{for } i = 2\ldots\nu : \mathrm{row}_{\nu+i} &:= \mathrm{row}_{\nu+i} - \mathrm{row}_{\nu+1} \\
\mathrm{col}_1 &:= \mathrm{col}_1 + \sum_{j=2}^{\nu}\mathrm{col}_j
\end{aligned}$$

be reduced to

$$[\lambda^2 - (a+d)\lambda + ad - cb]^{\nu-1}[\lambda^2 - (a+d+\nu f)\lambda - ad - cb + \nu fb - \nu fa] = 0$$

The roots and the conditions that they have negative real parts are

$$\lambda = \frac{1}{2}[a + d \pm \sqrt{(a+d)^2 + 4(cb - ad)}] < 0 \quad\Longrightarrow\quad cb < ad$$

$$\lambda = \frac{1}{2}[a + d + \nu f \pm \sqrt{(a+d+\nu f)^2 + 4(cb - ad + \nu f(b-a))}] < 0 \quad\Longrightarrow\quad cb + \nu fb < ad + \nu fa$$

By reintroducing (21) it is found that the sufficient and necessary condition that these equations only have negative roots is $\tilde{C} > B_0/2$. This same line of reasoning can be applied to all motor units with a similar result.

Since C_{nm} cannot exceed B_0 (from equation (4)) the limits on $\tilde{C}_{nm}$ for a stable single innervation state is

$$B_0/2 < \tilde{C}_{nm} < B_0 \tag{22}$$

Since $\tilde{C}_{nm}$ cannot be arbitrarily small it follows from (22) that there is an upper bound to the number of terminals that a given amount of presynaptic factor A_0 will allow a motor neuron to support. Using (12) – (15) we obtain

$$A_0 - \nu_n(\tilde{A} + \tilde{C}) = \frac{\delta\nu_n}{\gamma}\frac{\tilde{A}}{\tilde{C}} \quad \text{and} \quad \tilde{A} = \frac{\beta}{\alpha}\frac{1}{B_0 - \tilde{C}}$$

Combining these with $\tilde{C} > \frac{1}{2}B_0$ we arrive at an upper limit on the number of terminals ν_n

$$\nu_n = \frac{\alpha\gamma A_0\tilde{C}(B_0 - \tilde{C})}{\alpha\gamma\tilde{C}^2(B_0 - \tilde{C}) + \beta\gamma\tilde{C} + \beta\delta} < 2\frac{A_0}{B_0} \tag{23}$$

5.3 The importance of the value of A_0/B_0

Our analysis shows that there is an upper limit on the number of terminals that can be supported by a neuron in the stable state of single innervation (23). This number is proportional to the value of A_0/B_0. If therefore the value of A_0/B_0 is so low that the maximum number of terminals allowed is less than the number present initially, then terminals will retract – even in the absence of competition from other axons. Conversely, if the value A_0/B_0 is high enough, no intrinsic withdrawal may be needed.

This finding offers an explanation of the seemingly contradictory evidence obtained from different muscles concerning intrinsic withdrawal, by assuming that different muscles have different ratios of the initial number of terminals to the value of A_0/B_0 (fig 5).

Bennett & Robinson matched up the total amount of presynaptic and postsynaptic substance available, by setting $A_0/B_0 = M/N$. However, this seems a somewhat arbitrary decision. It seems unlikely that information about B_0, M and N would be available to each motor neuron to enable this equality to be maintained in, say, the partial denervation experiments, where the value of N is changed artificially. We chose the value of A_0/B_0 according to (23), to determine the maximum number of terminals per neuron. Intrinsic withdrawal will now occur only when the initial number of terminals violate (23). Note that elimination of terminals does not necessarily stop as soon as condition (23) is satisfied (see section 6.3).

6 Simulations

Since our analysis was restricted to examining the stable state we needed to confirm that the model reproduces the entire process of withdrawal. To do this we employed computer simulations.

In the simulated system there are as many muscle fibres and motor neurons as found in real muscles. Running simulations that model real muscles has the advantage that we can model cases that have been investigated experimentally and allows us to compare experimental findings with model behaviour with great ease.

Numerical solutions to the equations for A_{nm} and C_{nm} were obtained by approximating the differential equations with difference equations. The difference equations are solved by an iterative method. Throughout these simulations the timestep value $\Delta t = 0.001$ days was used. The choice of Δt was justified by the finding that a doubling of Δt only had negligible influence (in the order of 0.1%) on simulation results.

These full scale simulations are compute intensive. Simulating the development of a muscle consisting of 3500 fibres innervated by 25 motor neurons with an initial degree of polyinnervation of 5 for a few weeks (as in fig 2), takes ~ 4 hours on a SPARC (Sun 4/60) workstation.

6.1 Numerical values & initial innervation

When determining the initial pattern of innervation, there are two important distributions to consider.

Distribution of motor unit sizes Should all neurons have an equal number of collaterals or should there be a wide spread in the initial motor unit sizes? For the rat soleus a wide initial spread was assumed. In other simulations all motor units were initially approximately the same size.

Distribution of multiple innervation How should the number of terminals per endplate be distributed? As far as we know, this question has not been addressed experimentally, but theoretical arguments support a random distribution (Willshaw, 1981). We chose the number of terminals at each endplate from a normal distribution with a standard deviation less than the mean. This ensured that nearly all endplates were multiply innervated.

Once the terminals have been chosen, a starting value has to be assigned to A_{nm} and C_{nm} for each terminal nm. We have chosen $A_{nm} = 0$ for all terminals and for C_{nm} we used values drawn from a normal $\mathcal{N}(0.04, 0.005)$ distribution (ie mean 0.04 & standard deviation 0.005). It is important to introduce some kind of randomness into the system to break the symmetry. This is not a problem in the biological world since many factors introduce noise here. We set the threshold value at $\theta = 0.01$, but the exact value is unimportant since once terminals get below a certain size they will shrink continuously. Bennett & Robinson assume that the initial value of C_{nm} should be identical for all terminals. In their system, symmetry is broken only by the spread in initial motor unit sizes. We think our choice is biologically more reasonable.

In order to solve the equations numerically we need to specify the values of the constants and the ratio A_0/B_0. We arbitrarily set $B_0 = 1$ and determine the ratio A_0/B_0 by specifying A_0. The value of A_0/B_0 is chosen in accordance with physiological investigations of maximum motor unit size in each particular muscle type. The values of the rate constants α, β, γ and δ were adjusted empirically to mimic the time course of elimination found experimentally (fig 2). In all simulations the values were $\alpha = 45$, $\beta = 0.4$, $\gamma = 3.0$ and $\delta = 2.0$. The values have not been optimized in any systematic way, but our results do not seem to be very sensitive to exact parameter values.

We present simulations of 3 different muscles

The rat soleus muscle This muscle typically has $N = 25$ neurons, $M = 3500$ muscle fibres and an initial degree of polyinnervation of 5. The initial distribution of multiple innervation was drawn from a normal $\mathcal{N}(5, 1)$ distribution. The distribution of motor unit sizes contained a large spread (fig 3).

The mouse soleus muscle This muscle typically has $N = 20$ neurons, $M = 600$ muscle fibres and an initial degree of polyinnervation of 6. The initial distribution of multiple innervation was drawn from a $\mathcal{N}(6, 1)$ distribution. All motor units are roughly the same size initially.

The rat lumbrical muscle This muscle typically has $N = 12$ motor neurons, $M = 600$ at time of birth but $M = 960$ in the mature muscle. The initial amount of polyinnervation is 2.7 typically and so the initial distribution of multiple innervation was drawn from a $\mathcal{N}(2.7, 0.5)$ distribution.

In all our simulations we assumed that competition begins 3 days after birth.

6.2 Elimination of polyinnervation in rat soleus

Figure 2 shows how the number of innervated fibres changes over time in a rat soleus muscle. Our simulation results are plotted together with experimental findings (Brown *et al.*, 1976). The simulation results are close to the experimental data. In the same graph the number of fibres receiving <u>some</u> innervation is plotted. The fact that this curve stays very close to 3500 fibres show that only very few (if any) fibres become denervated.

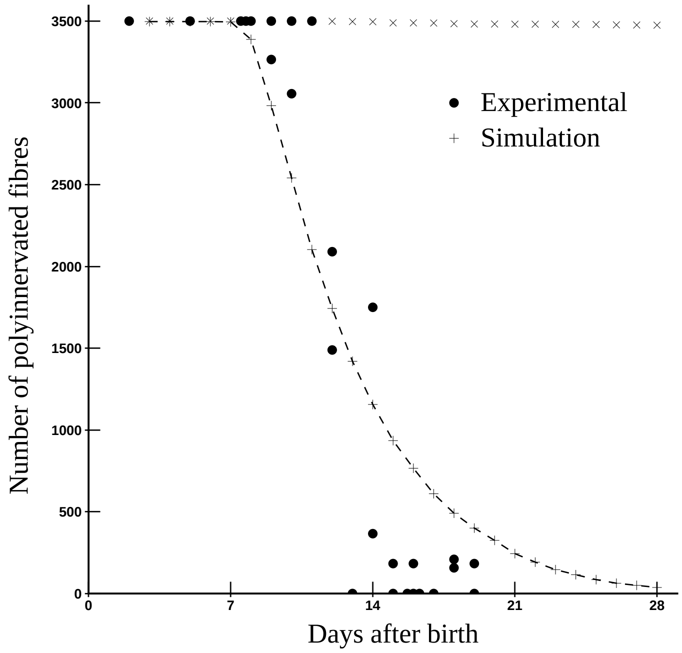


Figure 2: The time course of the elimination of polyinnervation in rat soleus muscle. There are $N = 25$ motor neurons innervating $M = 3500$ muscle fibres. The amount of polyinnervation falls rapidly during the second and third week after birth. Polyinnervation is virtually extinct in animals older than 4 weeks. Also shown in the same graph ($\times$) is the number of fibres in the simulation receiving <u>some</u> innervation, ie polyinnervation or single innervation. This shows that virtually all fibres that initially receive innervation are still innervated when polyinnervation has been withdrawn.

Figure 3 shows the distribution of motor unit sizes at two stages of development, from the simulation shown in fig 2. At birth we introduced a spread of ~ 8 in line with findings of Brown *et al.* (1976). During the course of elimination there is a marked reduction in this spread, to ~ 3, as also found by these authors. Note that the motor units that are initially largest end up being very small. This is an effect of the presynaptic sum rule.

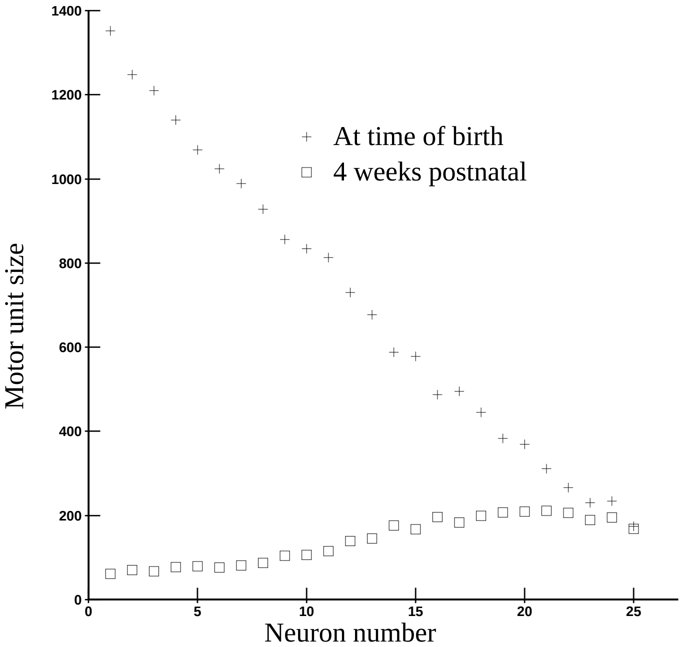


Figure 3: Simulation of rat soleus muscle. Reduction in the spread of motor unit size during withdrawal of polyinnervation. There are $N = 25$ motor neurons and $M = 3500$ muscle fibres. The spread in motor unit sizes is reduced substantially from ~ 8 to ~ 3. This corresponds to the reduction found experimentally.

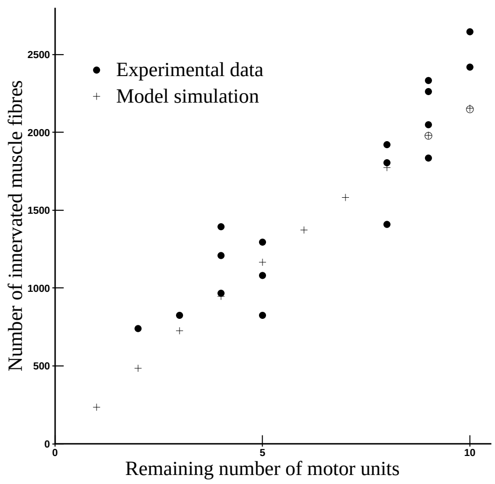
6.3 Partial denervation experiments

Experiments of partial denervation of rat soleus (Thompson & Jansen, 1977) and mouse soleus (Fladby & Jansen, 1987) both concluded that when the muscle was only partially innervated then the size of the motor units was independent of the number of the remaining motor neurons. This size was smaller than the average size in neonatal muscle but larger than the normal adult size. This suggests that motor units have a limited maximum size (as noted earlier).

Figure 4a and fig 4b show simulation results together with experimental data. Fig 4a shows the situation for the rat soleus together with the findings of Thompson & Jansen (1977). Fig 4b shows the same for mouse soleus and findings of Fladby & Jansen (1987). We used the experimental data to adjust our choice of the value of A_0/B_0. The values found were $A_0/B_0 = 175$ for the rat soleus muscle and $A_0/B_0 = 60$ for the mouse soleus.

To reach a value of A_0/B_0 for the rat lumbrical muscle we used a result by Ribchester (1987). He showed that if the muscle is innervated by 3 neurons or more then the muscle would be virtually completely innervated. The value of A_0/B_0 obtained through this observation is $A_0/B_0 = 200$.

Conflicting findings have been obtained when addressing the question of whether the loss of terminals could be ascribed solely to competition or whether some kind of intrinsic withdrawal plays an important role. A convenient way of addressing the problem is to remove the competition at the endplate by dissecting out all but a single motor axon. In the absence of intrinsic withdrawal



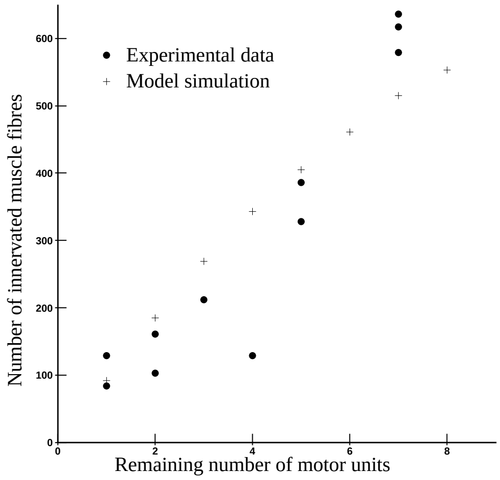


Figure 4: Degree of innervation in 6 week old muscles that were partially denervated at birth. The graphs show that there is an approximately proportional relationship between the number of motor units remaining after partial denervation and the number of fibres receiving innervation. This suggest that all motor units reach the same size. (a) Rat soleus has $M = 3500$ fibres and before partial denervation $N = 25$ motor neurons. Experimental findings from Thompson and Jansen (1977). (b) Mouse soleus has $M = 600$ fibres and before partial denervation $N = 20$ motor units. Experimental findings by Fladby and Jansen (1987).

there should be no reduction of the motor unit size in these circumstances.

Figure 5 shows how the unit size of a single motor unit changes over time in the absence of competition from other motor units. Betz *et al.* (1980) found no evidence for intrinsic withdrawal in the rat lumbrical muscle. On the contrary, Fladby & Jansen (1987) found a reduction of motor unit sizes in rat soleus that could not be ascribed solely to the effects of competition between terminals at an endplate. Our findings show that the combined presynaptic and postsynaptic competition can account for these seemingly contradictory results.

The simulation results confirm our analytical results that withdrawal in absence of competition from other motor units only takes place in certain cases depending on the value of A_0/B_0. It is important to note that there is no guarantee that a system that starts off with a large number of terminals will settle to a stable state as soon as (23) is satisfied. On the contrary, our simulations show that the system typically settles in a state with slightly fewer terminals than given by (23).

The final size of the single motor unit in the rat soleus is seen (fig 5) to be ~ 250. Equation (23) gives an overestimate of the theoretical maximum size which is 350.

6.4 Reinnervation of partially denervated muscle

Figure 6 refers to partial denervation in a mature lumbrical muscle. The simulation is designed to mimic experiments by (Ribchester, 1988). Partial denervation was introduced by removing one (the lateral plantar nerve) of the two nerves supplying the muscle. The remaining neurons

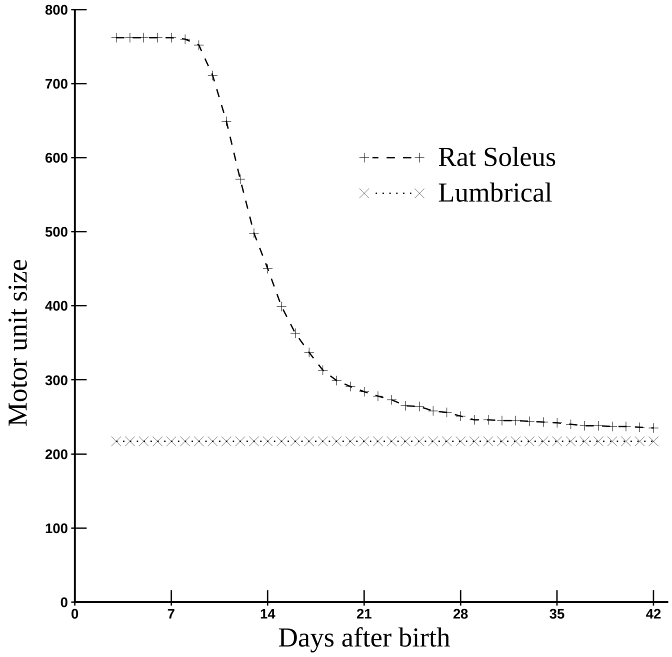


Figure 5: Simulation of the development of motor unit size in the absence of competition from other motor neurons. Muscles were partially denervated at birth, so that only a single motor unit survived in each case. The effect of intrinsic withdrawal is seen for the rat soleus muscle only. At 6 weeks the size of the soleus motor unit is close to its final value.

sprout and if there are at least three neurons left virtually all fibres get innervated. Around 2 weeks later the severed nerve returns to the muscle and competes with the remaining neurons. Ribchester reported that the reinnervating nerve was able to take over control of some muscle fibres from the remaining nerve. Our simulation shows the same ability. This is an important property of the model since it shows that the state of innervation is plastic even when single innervation has been reached.

7 Conclusion

Previous models for the elimination of superinnervation in developing muscle are based on various mechanisms of competition. We have argued that issues of implementation (Willshaw, 1981) and of the nature of the experimental results (Gouzé *et al.*, 1983) make these mechanisms inadequate. The dual constraint model (DCM) due to Bennett and Robinson (1989) employs two types of competition: for a presynaptic substance and for a postsynaptic substance.

We resolved certain questions about implementation that Bennett and Robinson left outstanding, and then performed perturbation analysis on the set of equations defining the improved model. This enabled us to derive the result that for the state of single innervation to be stable, the number of terminals made by each motor neuron cannot exceed a certain figure.

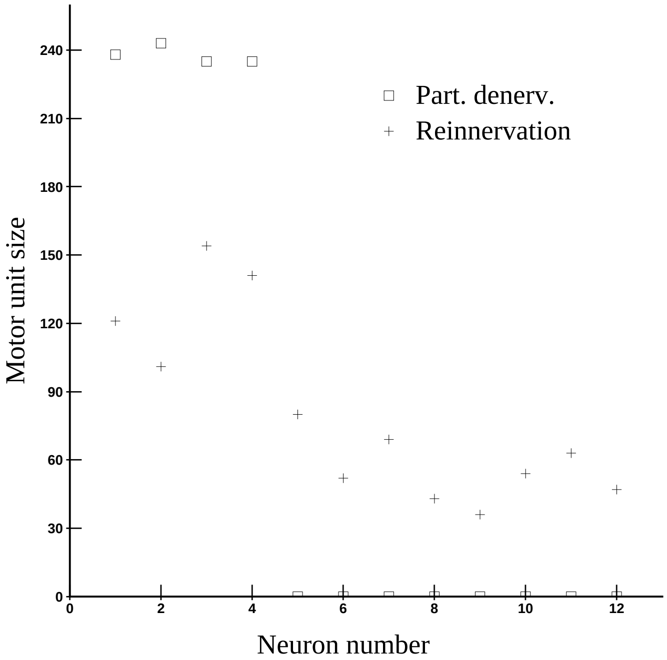


Figure 6: Plasticity of the pattern of connections in the simulation of adult rat lumbrical muscle. The adult muscle was partially denervated so that only 4 motor neurons remained, of the $N = 12$ that are normally present. These 4 motor units are assumed to have sprouted to innervate the entire muscle. Motor unit sizes are shown for the four intact units. When the injured motor neurons grew back, they recovered control over some muscle fibres, at the expense of the undamaged neurons. In the graph it is seen that all motor neurons have terminals, and motor units $1 - 4$ have shrunk.

This model mimics the experimental findings well and the underlying mechanisms are biologically plausible. Results from our analysis and our simulations showed that in all cases the system converged to a state of single innervation and the terminals of any particular motor neuron were all of the same size.

In some of Bennett and Robinson's smaller-scale simulations of their slightly different model, some endplates remained multiply innervated after many time steps. It is not clear to us whether these represent stable end-states and, if so, whether they are an artifact of the initial conditions that they chose.

In future work we will incorporate into the model the effect of activity and a explicit mechanism of sprouting. We will have to decide which of the biochemical reactions underlying the model should be made activity dependent. Sprouting can be thought of as the formation of new terminals into which small amounts of the presynaptic substance A that has not yet been converted to the binding complex C are released. Unlike other models (Gouzé *et al.*, 1983), the underlying chemical reactions of DCM are reversible and so the stable endstate of single innervation may not remain stable once sprouting occurs.

Our most important finding is that there is a maximum number of terminals that a motor neuron can maintain, which is determined by the ratio of the amount A_0 of presynaptic substance available per motor neuron to the amount B_0 of postsynaptic substance per endplate. Bennett and Robinson chose the value of this ratio to match up the *total* amount of presynaptic substance to the total of postsynaptic substance. This is biologically unjustifiable and is not necessary computationally. If this ratio is very large then the presynaptic conservation rule becomes inoperative; if it is so small that the maximum number of terminals that a motor neuron can maintain is less than the initial amount of polyinnervation, then terminals will necessarily withdraw, even in the absence of competition from other terminals; ie, there is intrinsic withdrawal. We set the value of A_0/B_0 in accord with the maximum number required by the results of neonatal partial denervation experiments.

Our analysis suggests that intrinsic withdrawal should not be regarded as a mechanism in its own right but rather a side effect of a general mechanism employed by the neuromuscular system to achieve similar motor unit sizes under a wide variety of conditions.

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