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Poly- and Mononeuronal Innervation in a Model for the Development of Neuromuscular Connections

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In the normal development of connections between motor neurons and muscle fibres, an initial stage of polyneuronal innervation is followed by withdrawal of connections until each muscle fibre is innervated by a single axon. However, polyneuronal innervation has been found to persist after prolonged nerve conduction block, in spite of the resumption of normal neuromuscular activity.

Here we analyse in detail a model proposed for the withdrawal of nerve connections in developing muscle, based on competition between nerve terminals. The model combines competition for a pre-synaptic resource with competition for a post-synaptic resource. Using bifurcation and phase space analysis, we show that polyneuronal innervation, as well as mononeuronal innervation, can be stable. The model acounts for the development of mononeuronal innervation and for persistent polyneuronal innervation after prolonged nerve conduction block, which appears as a consequence of the general competitive interactions operating during normal development.

1. Introduction

In many parts of the vertebrate nervous system, development involves a reduction in the number of axons innervating individual target cells (Purves & Lichtman, 1980). The best studied system is the innervation of mammalian skeletal muscle by its motor nerve (Redfern, 1970; Jansen & Fladby, 1990). During pre-natal development, the axons of the motor neurons grow towards their target muscle, and near the muscle each axon arborizes to innervate a large number of muscle fibres. At birth each muscle fibre is contacted by axon terminals from several different motor neurons, i.e. there is polyneuronal innervation. In the subsequent few weeks, axons lose some of their connections until each muscle endplate is innervated by a single axon. A similar sequence of events is found during reinnervation of adult muscle following injury to the motor nerve (McArdle, 1975; Brown *et al.*, 1976; Betz *et al.*, 1979).

The mechanisms for the withdrawal of connections in the neuromuscular junction are still poorly understood. The evidence suggests that this involves competition between nerve terminals both for a pre-synaptic resource and for a post-synaptic resource (Thompson & Jansen, 1977; Fladby & Jansen, 1987).

Several formal models have been proposed for the elimination of polyneuronal innervation

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during the development of neuromuscular connections (Willshaw, 1981; Gouzé *et al*., 1983; Bennett & Robinson, 1989; Elliott & Shadbolt, 1996). The so-called *dual constraint model* due to Bennett & Robinson (1989) has been shown (Rasmussen & Willshaw, 1993) to be superior to others. It can account for the development of single innervation as well as many other experimental findings, and has a biologically plausible implementation. Using perturbation analysis, Rasmussen & Willshaw (1993) have shown that single innervation is a stable state of the model.

In some of Bennett & Robinson's simulations, some muscle fibres remained polyneuronally innervated after many time steps. Neither they nor Rasmussen & Willshaw (1993) analysed whether these represent stable end states. Stable polyneuronal innervation has been found in partial denervation experiments after reinnervation and recovery from prolonged nerve conduction block (Brown *et al*., 1982; Barry & Ribchester, 1995).

In this paper we extend the analysis of the dual constraint model, employing bifurcation and phase space analysis. We examine whether polyneuronal innervation can be stable and whether the model can account for the experimental result of persistent polyneuronal innervation following earlier nerve conduction block.

In Section 2 we review the experimental findings concerning the normal development of neuromuscular connections, the evidence for competition therein, and the occurrence of persistent polyneuronal innervation. In Section 3 we describe the dual constraint model, largely following the description in Rasmussen & Willshaw (1993). In Section 4 we give our analysis of the model and study whether the model can account for persistent polyneuronal innervation.

2. Experimental Findings

2.1. NORMAL DEVELOPMENT

In any one muscle the amount of initial innervation varies from fibre to fibre with very few, if any, uninnervated muscle fibres. The average amount of initial innervation, expressed as the mean number of axons per endplate, also varies. In rat lumbrical muscle it is around three (Betz *et al*., 1979), and around six in the mouse soleus muscle (Fladby & Jansen, 1988). This state of polyneuronal innervation is transient and is removed during the first few weeks after birth. The extent of innervation can also be expressed in terms of *motor unit size*, which is the number of fibres contacted by a given motor axon. The variability in size amongst motor units can decrease substantially during the elimination of polyneuronal innervation. Brown *et al*. (1976) found that the ratio of the size of the largest motor unit to the smallest was reduced from eight initially to three in the mature muscle.

Initial high levels of polyneuronal innervation of individual endplates have also been described in amphibians (Morrison-Graham, 1983; Werle & Herrera, 1991). As in mammals, many terminals regress during subsequent development (Grinnell & Herrera, 1981). Yet in many frog muscles, for example, the competitive events do not lead to a complete elimination of polyneuronal innervation. In the adult cutaneous pectoris muscle, for instance, as many as a third of the endplates are still innervated by two different motor axons (Trussell & Grinnell, 1985).

2.2. PARTIAL DENERVATION EXPERIMENTS AND COMPETITION

In neonates and adults, muscles can be partially denervated by injuring some of the axons in the motor nerve. Reinnervation of the muscle by sprouting of intact axons and regeneration of damaged axons may then result in polyneuronally innervated muscle fibres (McArdle, 1975; Brown *et al*., 1981; Taxt, 1983; Barry & Ribchester, 1995). The subsequent elimination of polyneuronal innervation resembles that seen during normal post-natal development (McArdle, 1975; Brown *et al*., 1976; Betz *et al*., 1979). Partial denervation experiments are used to investigate the mechanisms involved in the elimination of polyneuronal innervation.

Following removal of some motor axons at birth, the average size of the remaining motor units after elimination of polyneuronal innervation is larger than normal (Thompson & Jansen, 1977; Fladby & Jansen, 1987). Individual motor axons appear to innervate more fibres as the result of the absence of other axon terminals. This suggests that there is a competitive process whereby terminals from different axons compete for control of the same endplate.

However, competition for the endplate alone (the post-synaptic site) cannot account for all findings. If there were only competition at the post-synaptic site, the elimination of terminals at each endplate would occur independently of the elimination at other endplates. Therefore post-synaptic competition alone cannot explain why large motor units reduce in size more than smaller ones, and that terminals at singly innervated fibres can withdraw (Fladby & Jansen, 1987), i.e. in the absence of competition from other terminals. The latter observation has led to the suggestion that there is a separate non-competitive mechanism of *intrinsic withdrawal*, by which a certain number of the initial connections are withdrawn regardless of competition (Thompson & Jansen, 1977; Fladby & Jansen, 1987).

After most of the motor units in the neonatal mouse soleus are removed, leading to incomplete innervation in the adult muscle, the average motor unit size found in the adult is independent of the remaining number of motor units (Fladby & Jansen, 1987). This suggests that there is a limited pre-synaptic capacity, so that each neuron can maintain only a limited number of terminals. A limited pre-synaptic capacity can explain withdrawal at singly innervated fibres and the reduction in variability of motor unit sizes during elimination of polyneuronal innervation, since terminals of larger motor units would be weaker and therefore less competitive than those of smaller motor units.

Taken together, the experimental data suggest that there is both competition for a pre-synaptic resource and competition for a post-synaptic resource. Post-synaptic competition is needed to achieve single innervation and pre-synaptic competition to account for many other experimental findings.

2.3. PERSISTENT POLYNEURONAL INNERVATION

Blocking nerve conduction in the motor nerve, either during normal development or reinnervation, increases the proportion of polyneuronally innervated muscle fibres, either by stimulating nerve sprouting and/or by reducing elimination of terminals (Thompson *et al*., 1979; Duxson, 1982; Taxt, 1983; Ribchester, 1993).

Prolonged nerve conduction block was used by Barry & Ribchester (1995) to increase the initial extent of polyneuronal innervation of rat lumbrical muscle after partial denervation. Following recovery from the block, activity resumes, and although some connections subsequently regress, many muscle fibres remain polyneuronally innervated, in spite of the resumption of normal neuromuscular activity. Thus stable polyneuronal innervation appears as a consequence of earlier nerve conduction block. These results suggest that muscle paralysis provides an environment in which polyneuronal innervation can become consolidated. Persistent polyneuronal innervation is also found in mouse tensor fasciae latae muscle after neonatal paralysis of motor innervation (Brown *et al*., 1982). However, polyneuronal innervation is almost completely eliminated in mouse gluteus muscles treated in the same way (Brown *et al*., 1982). Persistent polyneuronal innervation has also been described in rat levator ani muscle after neonatal treatment with testosterone (Lubischer *et al*., 1992).

3. Dual Constraint Model

Several formal models have been proposed for the elimination of polyneuronal innervation during the development of neuromuscular connections. The so-called dual constraint model due to Bennett & Robinson (1989), which combines competition for a pre-synaptic resource with competition for a post-synaptic resource, has been shown to be superior to others with only one type of competition (Rasmussen & Willshaw, 1993). It accounts for many anatomical and physiological findings, and has a biologically plausible implementation. In the model, a reversible reaction between the pre-synaptic substance A and the post-synaptic

substance B produces binding complex C. This binding complex is essential to the maintenance of a terminal; the size of a terminal is assumed to be directly proportional to the amount of C. The reversible reaction takes place in the synaptic cleft, where pre-synaptic molecules are located in the terminal membrane of a motor axon, and post-synaptic molecules are found in the endplate membrane of a muscle fibre. There are N motor neurons and M muscle fibres. A motor neuron is indexed by n, a muscle fibre by m and a terminal by nm. The reversible reaction takes one molecule of both A and B to produce one of C

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

The rate of the forward reaction is taken to be proportional to the product of the amounts of A_{nm}, B_m and a fixed power $\mu > 0$ of the amount of C_{nm}. The rate of the backward reaction is taken to be proportional to the amount of C_{nm}

$$\frac{\mathrm{d}C_{nm}}{\mathrm{d}T} = \alpha A_{nm} B_m C_{nm}^{\mu} - \beta C_{nm}, \tag{2}$$

where α and β are rate constants. Including C_{nm}^{μ} in the rate of the forward reaction incorporates a positive feedback: larger terminals (i.e. terminals that possess a larger amount of C_{nm}) favour the forward reaction and so can become larger still. The justification given by Bennett & Robinson (1989) for including this positive feedback is that electrical activity in the nerve terminal could produce electromigration of molecules B in the endplate. The result is that larger terminals will have an ability to attract molecules of B towards the region immediately under the terminal, thus favouring the forward reaction at these terminals. Including C_{nm}^{μ} is needed to achieve single innervation (see Section 4.4). We choose $\mu = 1$.

We will now derive conservation equations for A and B and express A_{nm} and B_m in terms of C_{nm}. Each motor neuron n has a fixed amount A_0 of pre-synaptic 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

$$A_0 = A_n + \sum_{j=1}^{M} A_{nj} + \sum_{j=1}^{M} C_{nj}. \tag{3}$$

The amount of unbound pre-synaptic substance A_{nm} is assumed to be proportional to (i) the size of the terminal C_{nm} and (ii) the amount of pre-synaptic factor in the cell soma A_n, yielding

$$A_{nm} = KC_{nm}A_n, \tag{4}$$

where K is a constant. Bennett & Robinson (1989) do not discuss what process could give rise to the distribution of A given by eqn (4). Rasmussen & Willshaw (1993) suggest a scheme of transport mechanisms that could give rise to such a distribution. Their scheme involves anterograde transport of A down the axon and retrograde transport of A from the terminal.

Using eqns (3) and (4)

$$A_{nm} = KC_{nm}\frac{A_0 - \Sigma_{j=1}^{M} C_{nj}}{1 + K\,\Sigma_{j=1}^{M} C_{nj}}. \tag{5}$$

Each muscle fibre m has a fixed amount B_0 of post-synaptic 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}, in the terminal membrane. The conservation equation for B is

$$B_0 = B_m + \sum_{i=1}^{N} C_{im}, \tag{6}$$

and therefore

$$B_m = B_0 - \sum_{i=1}^{N} C_{im}. \tag{7}$$

Note that in all equations the sums taken over neurons and muscle fibres are only over terms for which terminals exist since not all neurons necessarily have terminals at all muscle fibres. Introducing eqns (5) and (7) in (2) gives a set of differential equations for how C_{nm} changes over time.

Rasmussen & Willshaw (1993) analysed this model using perturbation analysis and simulations. They showed that the single innervation state is stable, and that there is an upper limit on the number of terminals that can be supported by each motor neuron. This limit was shown to be proportional to the value of A_0/B_0. So if the initial amount of polyneuronal innervation is larger than this limit, then necessarily terminals will withdraw, even in the absence of competition, i.e. there is intrinsic withdrawal. An important result from their study is that therefore intrinsic withdrawal should not be regarded as a separate non-competitive mechanism (Thompson & Jansen, 1977; Fladby & Jansen, 1987) but rather as a side effect of the competitive mechanism.

Rasmussen & Willshaw (1993) did not analyse, nor did Bennett & Robinson (1989) whether polyneuronal innervation can be a stable state of the model.

4. Our Analysis

In order to reduce the number of parameters, we convert the equations into a non-dimensional form by choosing the following non-dimensional quantities

$$c_{nm} = \frac{C_{nm}}{B_0},\ a_{nm} = \frac{A_{nm}}{B_0},\ b_m = \frac{B_m}{B_0},\ t = T\beta. \quad (8)$$

Equations (2), (5) and (7) in non-dimensional form are

$$\frac{\mathrm{d}c_{nm}}{\mathrm{d}t} = \gamma a_{nm} b_m c_{nm} - c_{nm} \quad (9)$$

$$a_{nm} = k c_{nm} \frac{a_0 - \Sigma_{j=1}^{M} c_{nj}}{1 + k \Sigma_{j=1}^{M} c_{nj}} \quad (10)$$

$$b_m = 1 - \sum_{i=1}^{N} c_{im}, \quad (11)$$

with non-dimensional parameters

$$a_0 = \frac{A_0}{B_0},\ \gamma = \frac{\alpha}{\beta} B_0^2,\ k = KB_0. \quad (12)$$

The sums taken over neurons and muscle fibres in eqns (10) and (11) are only over terms for which terminals exist. Note that a_{nm}, b_m and c_{nm} must always be positive, i.e. valid values of c_{nm} (including initial values) are such that $\Sigma_{j=1}^{M} c_{nj} < a_0$ and $\Sigma_{i=1}^{N} c_{im} < 1$ with $c_{nm} > 0$, for all n and m.

At equilibrium $\mathrm{d}c_{nm}/\mathrm{d}t = 0$, i.e.

$$c_{nm} = 0$$

or (13)

$$\gamma k c_{nm} \frac{a_0 - \Sigma_{j=1}^{M} c_{nj}}{1 + k \Sigma_{j=1}^{M} c_{nj}} \left(1 - \sum_{i=1}^{N} c_{im}\right) - 1 = 0.$$

Consider the case where there is a single muscle fibre m (i.e. $\Sigma_{j=1}^{M} c_{nj} = c_{nm}$ for all n) and $v \leqslant N$ neurons which have terminals on this muscle fibre. Taking the second solution in eqn (13) for all v terminals defines a system of v equations with v unknowns c_{nm}. A number of equations may be satisfied simultaneously, i.e. polyneuronal innervation may be possible. In the general case the second solution defines a system of at most $N \times M$ equations with as many unknowns c_{nm}. For a given m there may be a number of equations satisfied simultaneously, i.e. there may be polyneuronal innervation.

We further analyse the model using phase space analysis, bifurcation analysis and numerical integration, for which we use the computer programme GRIND (De Boer, 1983). We deliberately use small numbers of neurons and fibres to obtain a better understanding of the model. We first consider configurations of the model that allow us to study competition for post-synaptic substance and competition for pre-synaptic substance in isolation of each other (Sections 4.1 and 4.2, respectively). We then consider configurations where both types of competition take place at the same time (Section 4.3). The basic model configurations studied are given in Fig. 1. In Section 4.4 we study whether the model can account for the experimental result of persistent polyneuronal innervation following earlier nerve conduction block.

4.1. COMPETITION FOR POST-SYNAPTIC SUBSTANCE ONLY

We study the configuration where there are two motor neurons which both contact the same single muscle fibre [2n1m-model, Fig. 1(a)], i.e.

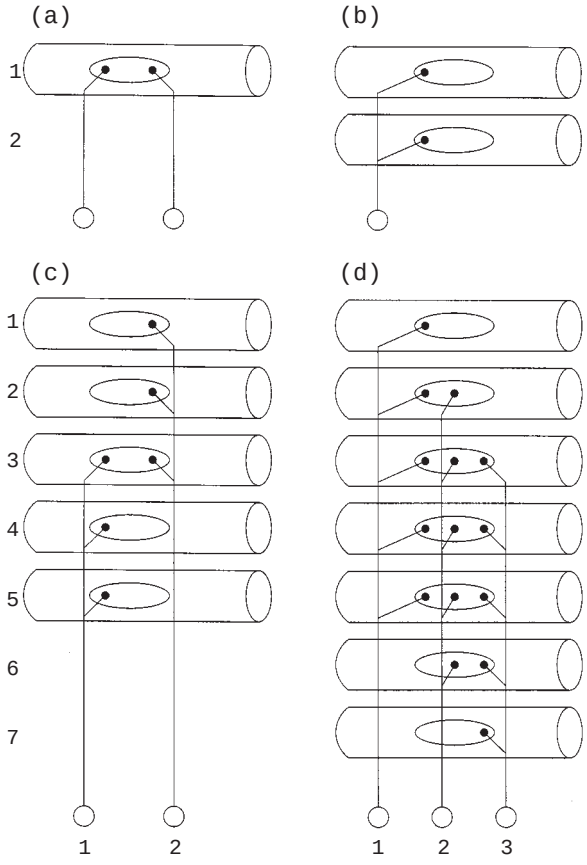

FIG. 1. Initial pattern of innervation of the various model configurations we studied. The configurations have different numbers of motor neurons (N) and muscle fibres (M). Each muscle fibre has a single endplate: (a) 2n1m-model: $N = 2$, $M = 1$; (b) 1n2m-model: $N = 1$, $M = 2$; (c) 2n5m-model: $N = 2$, $M = 5$; (d) 3n7m-model: $N = 3$, $M = 7$.

the initial degree of polyneuronal innervation is two. This is the simplest configuration where there is competition for post-synaptic substance only. The equations for the 2n1m-model are:

$$\frac{dc_{11}}{dt} = \gamma k c_{11} \frac{a_0 - c_{11}}{1 + kc_{11}} (1 - c_{11} - c_{21}) c_{11} - c_{11} \tag{14}$$

$$\frac{dc_{21}}{dt} = \gamma k c_{21} \frac{a_0 - c_{21}}{1 + kc_{21}} (1 - c_{11} - c_{21}) c_{21} - c_{21} \tag{15}$$

The bifurcation diagram in Fig. 2(a) depicts the equilibrium points of the whole model drawn on the (a_0, c_{21}) plane, for given values of γ and k. A bifurcation occurs when there is a change in the number or the nature of equilibrium points. The values of a_0 where this occurs are denoted by p, q, r and s. For $a_0 < p$ none of the terminals can be maintained in equilibrium. The only equilibrium point is ($c_{11} = 0$, $c_{21} = 0$) (no innervation), which is stable. As a_0 increases, new equilibrium points appear. At $a_0 = p$ a saddle-node bifurcation (= fold bifurcation) takes place. Since the system is symmetrical, four new equilibrium points appear, two stable and two unstable equilibrium points [Fig. 2(b)]. The unstable equilibrium points are saddles, the stable equilibrium points are stable nodes. These two nodes are the equilibrium points where either of the two terminals is present (single innervation). At $a_0 = q$ a saddle-node bifurcation takes place where two unstable equilibrium points appear, a saddle and an unstable node [Fig. 2(c)]. At $a_0 = r$ a subcritical pitchfork bifurcation takes place. Here the saddle that appeared at the bifurcation at $a_0 = q$ becomes a stable node while at the same time two new saddles appear [Fig. 2(d)]. This stable node is the equilibrium point where both terminals coexist (polyneuronal innervation). At $a_0 = s$ there is another subcritical pitchfork bifurcation at which the two saddles disappear and the stable node becomes a saddle again [Fig. 2(e)].

Thus for $r < a_0 < s$ the system has four stable equilibria: one polyneuronal innervation equilibrium, two single innervation equilibria, and one where there is no innervation. Which equilibrium will be reached in any particular situation will depend on the initial values of c_{11} and c_{21} (Fig. 3). In Fig. 4(a) the parameter region for which there are four stable equilibria, i.e. the region bounded by the pitchfork bifurcations, is drawn in the full (k, a_0, γ) space. Within this region, the sizes of the basins of attraction of the different equilibria are sensitive to the values of k, a_0 and γ. The higher k, γ or a_0, the larger the relative size of the basin of attraction of the polyneuronal innervation equilibrium, and the smaller the basins of attraction of the no innervation and single innervation equilibria. In Fig. 4(b) the saddle-node bifurcation that occurs at $a_0 = p$ in Fig. 2(a) is followed through the (k, a_0) parameter space for different values of γ. These lines define where the single innervation equilibria appear in the parameter space.

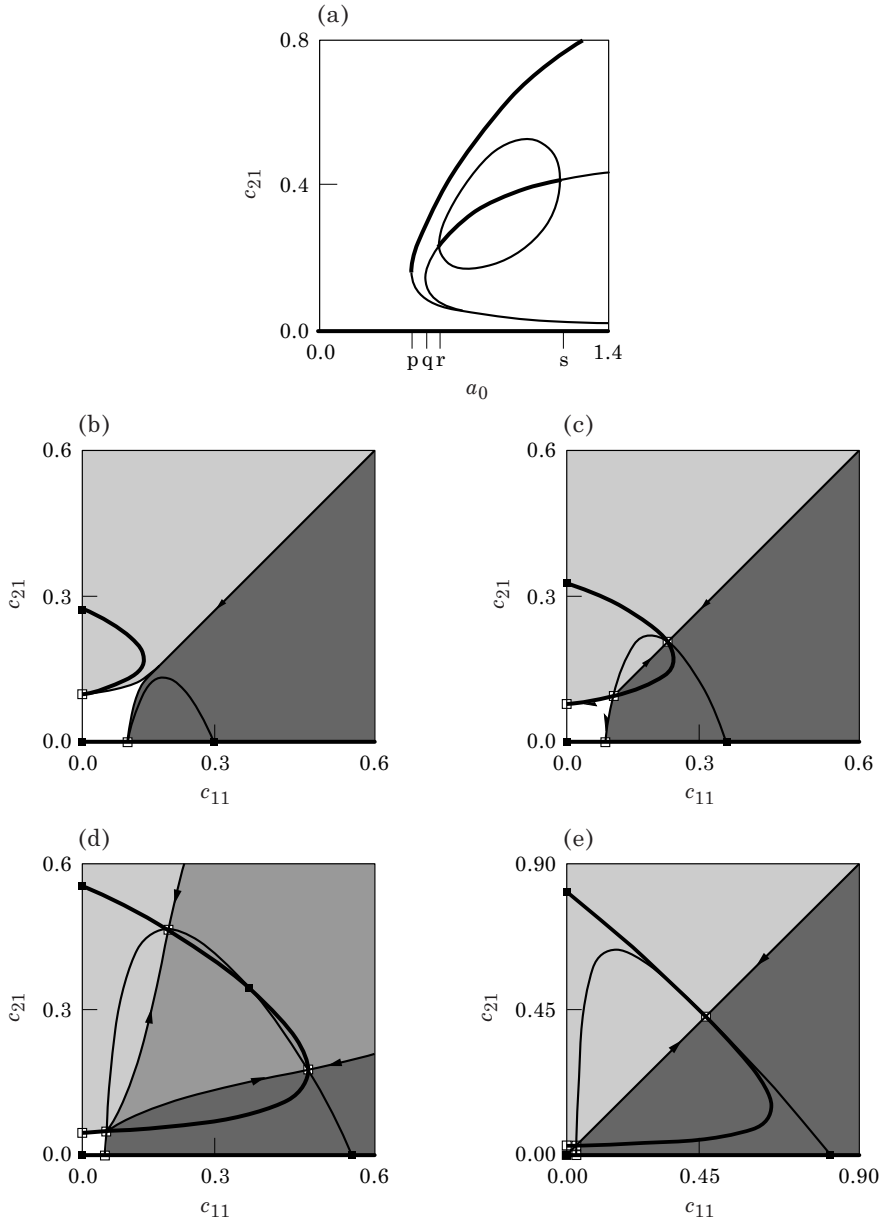

FIG. 2. Bifurcation and phase space analysis of the 2n1m-model. In all figures, $\gamma = 17$, $k = 2$: (a) bifurcation diagram. The curves depict the equilibrium points of the whole system (i.e. $dc_{11}/dt = dc_{21}/dt = 0$) drawn on the ($a_0$, c_{21}) plane. The bold lines indicate stable equilibrium points, the thin lines unstable ones; (b–e) the bold lines depict the solutions of $dc_{21}/dt = 0$, the thin lines the solutions of $dc_{11}/dt = 0$, i.e. they are the nullclines of c_{21} and c_{11}, respectively. Intersection points of these lines are equilibrium points; (■) stable equilibrium points and (□) unstable ones. Also drawn are the stable manifolds of the saddle points, which are the lines separating the basins of attraction of the stable equilibrium points. The arrows indicate the direction of flow on these manifolds; trajectories that start exactly on such a manifold converge to a saddle point, all other ones converge to a stable equilibrium. The white area is the basin of attraction of the equilibrium where $c_{11} = c_{21} = 0$ (no innervation), the light grey area that of the equilibrium where $c_{21} > 0$ and $c_{11} = 0$ (single innervation), the dark grey area that of the equilibrium where $c_{11} > 0$ and $c_{21} = 0$ (single innervation), and the intermediate grey area that of the equilibrium where both $c_{11} > 0$ and $c_{21} > 0$ (polyneuronal innervation). The nullcline pictures are drawn for different values of a_0: (b) $p < a_0 < q$, $a_0 = 0.5$; (c) $q < a_0 < r$, $a_0 = 0.55$; (d) $r < a_0 < s$, $a_0 = 0.8$; (e) $a_0 > s$, $a_0 = 1.3$.

The case with just two neurons is an example of the more general case. For instance, for an initial polyneuronal innervation of five (5n1m-model), there are stable polyneuronal innervation equilibria in which two, three, four or all five terminals are present [Fig. 5(a)]. The larger

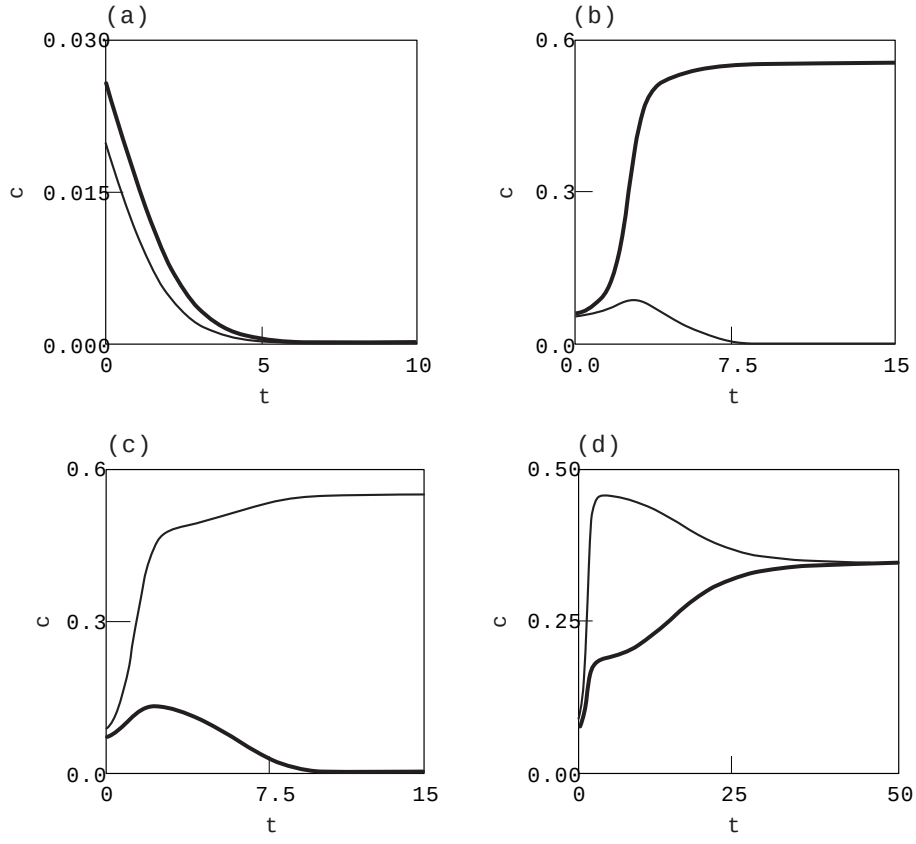

FIG. 3. Runs of the 2n1m-model starting from different initial conditions. Parameters as in Fig. 2(d). Bold line: c_{21}; thin line: c_{11}: (a) $c_{11} = 0.02$, $c_{21} = 0.026$; (b) $c_{11} = 0.054$, $c_{21} = 0.06$; (c) $c_{11} = 0.09$, $c_{21} = 0.071$; (d) $c_{11} = .0878$, $c_{21} = 0.074$.

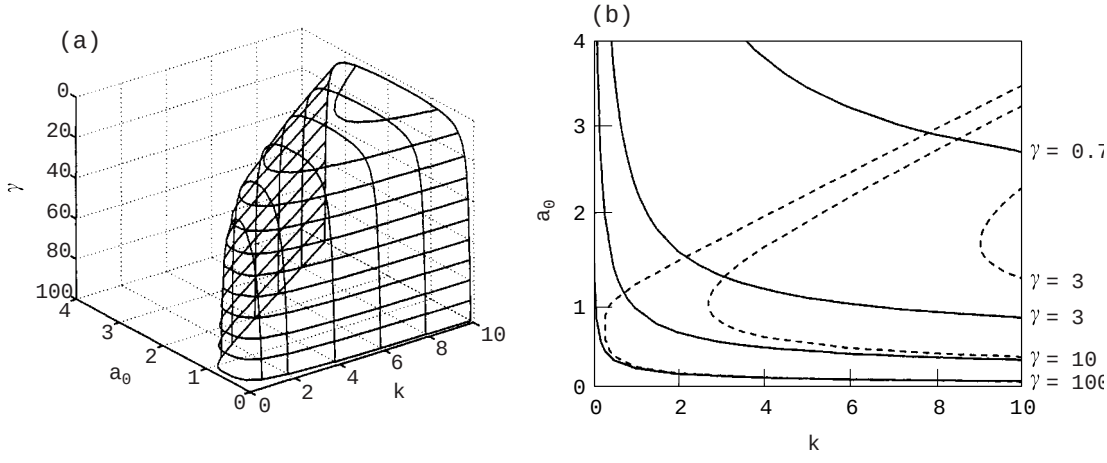

FIG. 4. Bifurcation analysis of the 2n1m-model: (a) region of the parameter space bounded by pitchfork bifurcation lines within which four stable equilibria exist: two single innervation equilibria, one polyneuronal innervation equilibrium, and one equilibrium where there is no innervation. See further text; (b) saddle-node bifurcation lines (——) together with pitchfork bifurcation lines (– – –). For $\gamma = 100$, $\gamma = 10$, $\gamma = 3$ and $\gamma = 0.75$. For $\gamma = 0.75$ the pitchfork bifurcation does not occur for the parameter region shown. In the region of the parameter space at the left-hand side of a solid line, no innervation is the only stable state; the region at the right-hand side has also the single innervation equilibria. The region at the right-hand side of a dashed line has, in addition, the stable polyneuronal innervation equilibrium.

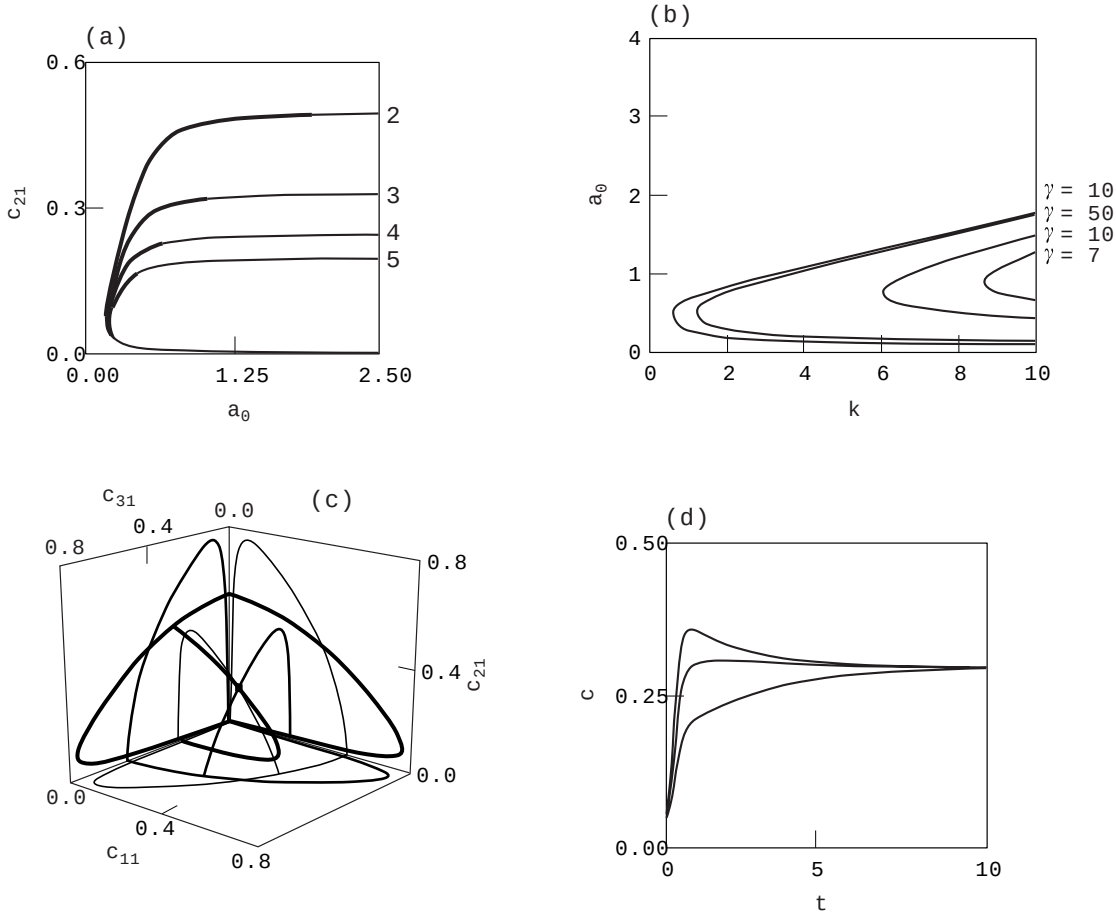

FIG. 5. Analysis of model configurations with more than one neuron and one muscle fibre: (a) bifurcation diagram of the 5n1m-model. Parameters: $\gamma = 50$, $k = 4$. The bold and thin lines indicate stable and unstable equilibrium points, respectively, of the whole system (i.e. all $dc_{nm}/dt = 0$) drawn on the (a_0, c_{21}) plane. For clarity, only branches are shown where polyneuronal innervation equilibria can be stable. The numbers (2–5) indicate the branches where, respectively, two, three, four and five terminals coexist in equilibrium. Note that the case with two terminals is identical to the 2n1m-model; (b) pitchfork bifurcation lines defining the region of parameter space (right-hand side of these lines) where three terminals can coexist in a stable equilibrium, for $\gamma = 100$, $\gamma = 50$, $\gamma = 10$ and $\gamma = 7$; (c) nullclines of the 3n1m-model. The thin, thick and medium thick lines are, respectively, the nullclines of c_{11} (i.e. solutions of $dc_{11}/dt = 0$), c_{21} ($dc_{21}/dt = 0$) and c_{31} ($dc_{31}/dt = 0$). They are drawn on the three side planes and there where they all intersect. The intersection point (■) is the stable equilibrium where all three terminals are present. Parameters: $\gamma = 50$, $k = 4$, $a_0 = 0.6$; (d) a run of the 3n1m-model starting from $c_{11} = 0.05$, $c_{21} = 0.06$ and $c_{31} = 0.07$. Parameters: $\gamma = 50$, $k = 4$, $a_0 = 0.6$.

the number of terminals in equilibrium, the smaller is the parameter region in which polyneuronal innervation is stable.

4.2. COMPETITION FOR PRE-SYNAPTIC SUBSTANCE ONLY

We study the configuration where there is one motor neuron with two terminals which each contact a different muscle fibre, i.e. the initial motor unit size is two [1n2m-model, Fig. 1(b)]. This is the simplest configuration where there is competition for pre-synaptic substance only. The equations for the 1n2m-model are:

$$\frac{dc_{11}}{dt} = \gamma k c_{11} \frac{a_0 - c_{11} - c_{12}}{1 + kc_{11} + kc_{12}} (1 - c_{11}) c_{11} - c_{11} \tag{16}$$

$$\frac{dc_{12}}{dt} = \gamma k c_{12} \frac{a_0 - c_{11} - c_{12}}{1 + kc_{11} + kc_{12}} (1 - c_{12}) c_{12} - c_{12} \tag{17}$$

In Fig. 6(a) we give the bifurcation diagram for the 1n2m-model.

values of a_0 where a bifurcation occurs are denoted by p, q and r. For $a_0 < p$ the only equilibrium point is ($c_{11} = 0$, $c_{12} = 0$). At the saddle-node bifurcation at $a_0 = p$, two saddles and two stable nodes appear [Fig. 6(b)]. The two nodes are the equilibrium points where either of the two terminals is present, i.e. where the motor unit size is one. At $a_0 = q$ a saddle-node bifurcation takes place where a saddle and an unstable node appear [Fig. 6(c)]. At the subcritical pitchfork bifurcation at $a_0 = r$ this saddle becomes a stable node while two new saddles appear [Fig. 6(d)]. The stable node is the equilibrium point where both terminals coexist, i.e. where the motor unit size is two.

Thus for $a_0 > r$ the system has four stable equilibria: one where the motor units size is two, two where the motor units size is one, and one where all terminals are absent. In Fig. 7 the saddle-node bifurcation at $a_0 = p$ and the pitchfork bifurcation at $a_0 = r$ is followed through the (k, a_0) parameter space for different values of γ. The pitchfork bifurcation line defines the region where there are four stable equilibria.

The case with a motor unit size of just two is an example of the more general case. For instance, for an initial motor unit size of five (1n5m-model), there are stable equilibria in which two, three, four or all five terminals are present (Fig. 8). The larger the value of a_0, the

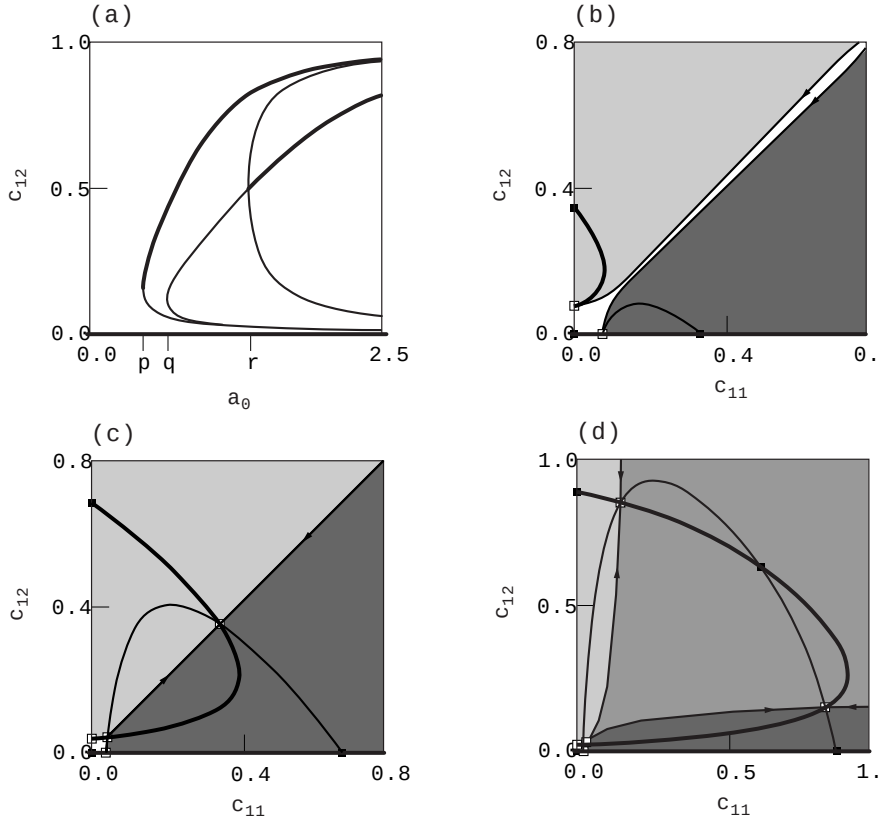

FIG. 6. Bifurcation and phase space analysis of the 1n2m-model. In all figures, $\gamma = 17$, $k = 2$: (a) the curves depict the equilibrium points of the whole system (i.e. $dc_{11}/dt = dc_{12}/dt = 0$) drawn on the ($a_0$, c_{12}) plane. The bold lines indicate stable equilibrium points, the thin lines unstable ones; (b–d) the bold and thin lines are the nullclines of c_{12} ($dc_{12}/dt = 0$) and c_{11} ($dc_{11}/dt = 0$), respectively. Intersection points of these lines are equilibrium points; (■) stable equilibrium points and (□) unstable ones. Also drawn are the stable manifolds of the saddle points, which separate basins of attraction. The arrows indicate the direction of flow on these manifolds. The white area is the basin of attraction of the equilibrium where $c_{11} = c_{12} = 0$ (no innervation), the light grey area that of the equilibrium where $c_{12} > 0$ and $c_{11} = 0$ (motor unit size one), the dark grey area that of the equilibrium where $c_{11} > 0$ and $c_{12} = 0$ (motor unit size one), and the intermediate grey area that of the equilibrium where both $c_{11} > 0$ and $c_{12} > 0$ (motor unit size two). The nullcline pictures are drawn for different values of a_0: (b) $p < a_0 < q$, $a_0 = 0.56$; (c) $q < a_0 < r$, $a_0 = 1.0$; (d) $a_0 > r$, $a_0 = 1.7$.

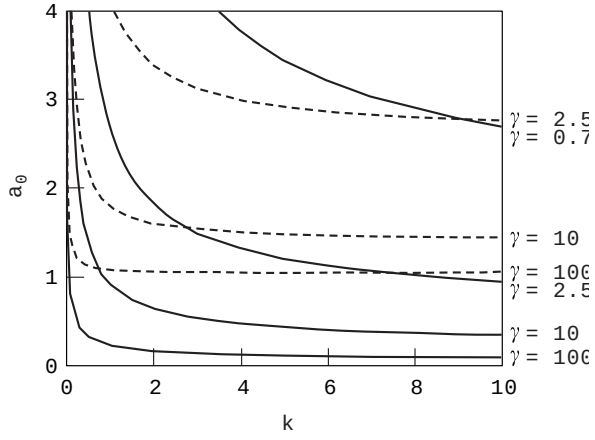

FIG. 7. Bifurcation analysis of the 1n2m-model: (——): saddle-node bifurcation lines; (– – –) pitchfork bifurcation lines. These lines are drawn for $\gamma = 100$, $\gamma = 10$, $\gamma = 2.5$ and $\gamma = 0.75$. For $\gamma = 0.75$ the pitchfork bifurcation does not occur in the parameter region shown. For a given value of γ, the region of the parameter space at the left-hand side of the corresponding solid line has as equilibrium only the state where all terminals are absent. The region at the right-hand side has also the equilibria where the motor unit size is one. At the right-hand side of the corresponding dashed line there is, in addition, the stable equilibrium where the motor unit size is two.

more terminals can coexist in a stable equilibrium, i.e. the larger the motor unit size can be.

4.3. COMPETITION FOR BOTH PRE- AND POST-SYNAPTIC SUBSTANCE

We study polyneuronal innervation in configurations of the model where competition for pre- and post-synaptic substance occurs at the same time. In the first example, there are five muscle fibres and two neurons, each of which initially contact three fibres (i.e. motor unit size is three), whereby one of the fibres is polyneuronally innervated [2n5m-model, Fig. 1(c)]. Polyneuronal innervation in the initial configuration can be stable, as well as in configurations where both neurons have motor unit size two or one (the latter is the 2n1m-model) (Fig. 9). It appears that both neurons must have the same motor unit size in order for polyneuronal innervation to be stable. Also, the higher the motor unit size, the larger the value of γ (and/or k) and a_0 should be for polyneuronal innervation to be stable (Fig. 9). For each particular parameter setting, the initial values of c_{nm} determine which of the possible stable innervation patterns will be reached.

In the second, larger example there are seven muscle fibres and three neurons (3n7m-model). The initial pattern of innervation is depicted in Fig. 1(d). Polyneuronal innervation can be stable, and a few possible configurations are shown in Fig. 10(a–c). Which innervation pattern will be reached depends on the initial values of c_{nm}. For polyneuronal innervation to be stable, it appears that neurons sharing a fibre must have the same motor unit size and that a neuron cannot be involved in the polyneuronal innervation of more than one fibre. Polyneuronal innervation is robust to small random differences among the terminals with respect to the values of k, a_o and γ.

To summarize our results so far, we have shown that polyneuronal and single innervation states can both be stable for a given parameter setting if the value of a_0 is not too large or too small (range depends on the values of k and γ). Which state will be reached in any specific situation depends on the initial amounts of binding complex, c_{nm}, and on the sizes of the basins of attraction of the different states, which are sensitive to the values of a_0, k and γ.

4.4. POLYNEURONAL INNERVATION EXPERIMENTS

We show that the model can account for the experimental finding that polyneuronal innervation can be maintained after nerve conduction block or other types of treatment (Brown *et al.*,

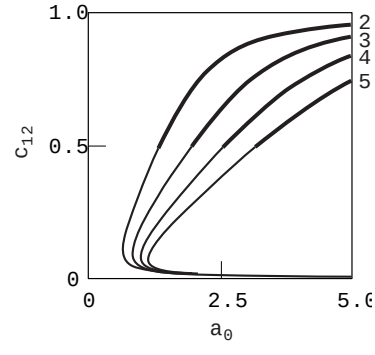

FIG. 8. Bifurcation diagram of the 1n5m-model. Parameters: $\gamma = 17$, $k = 2$. The bold and thin lines indicate stable and unstable equilibrium points, respectively, of the whole system (i.e. all $dc_{nm}/dt = 0$) drawn on the (a_0, c_{12}) plane. For clarity, only branches are shown where a motor unit size greater than one can be stable. The numbers (2–5) indicate the branches with a motor unit size of, respectively, two, three, four and five. Note that the case with two terminals is identical to the 1n2m-model.

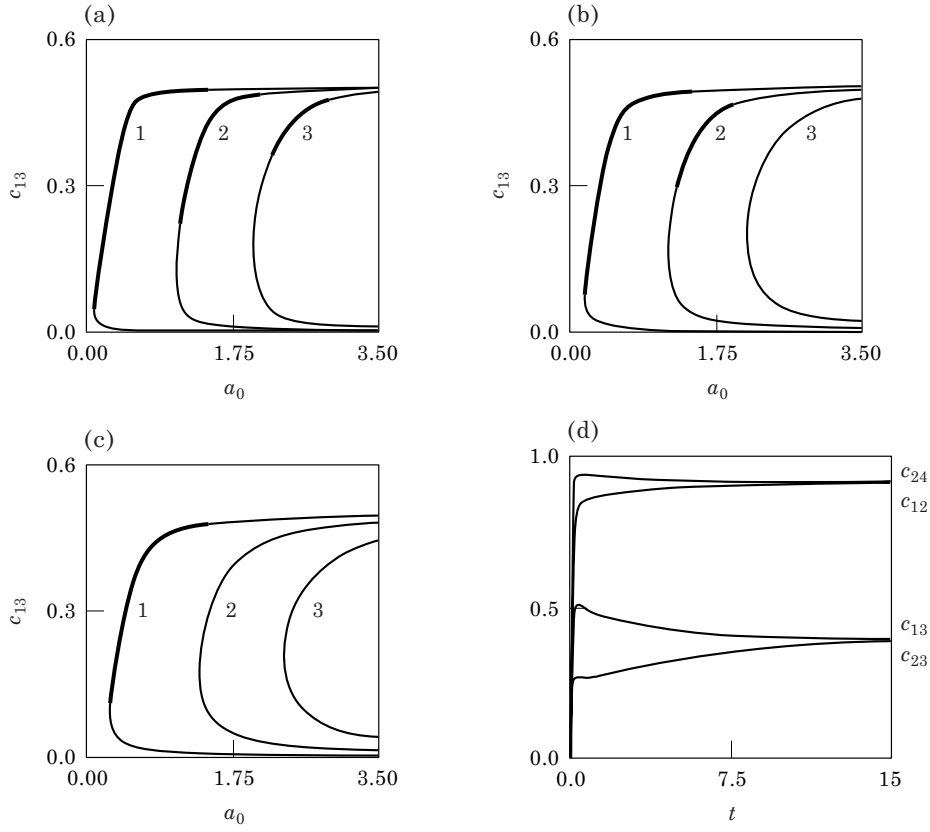

FIG. 9. (a–c) Bifurcation diagrams of the 2n5m-model for different values of γ. In all figures, $k = 2$. The bold and thin lines indicate stable and unstable equilibrium points, respectively, of the whole system (i.e. all $dc_{nm}/dt = 0$) drawn on the (a_0, c_{13}) plane. For clarity, only branches are shown where polyneuronal innervation can be stable. In each figure, the numbers (1–3) indicate the branches where the motor unit size of both neurons is, respectively, one, two and three: (a) $\gamma = 250$; (b) $\gamma = 100$; (c) $\gamma = 50$; (d) run with parameters $\gamma = 100$, $k = 2$, $a_0 = 1.5$. Initial conditions: $c_{12} = 0.05$, $c_{13} = 0.06$, $c_{23} = 0.07$, $c_{24} = 0.08$ and all other $c_{nm} = 0$.

1982; Lubischer *et al*., 1992; Barry & Ribchester, 1995). In terms of the model, blocking electrical activity in the motor nerve means that the suggested positive effect of electrical activity on the formation of binding complex C at the terminal (Section 3) is no longer operative, i.e. $\mu = 0$ in eqn (2). The non-dimensional form of eqn (2) now becomes, substituting eqns (10) and (11)

$$\frac{dc_{nm}}{dt} = c_{nm}\left(\gamma^* k \frac{a_0 - \sum_{j=1}^{M} c_{nj}}{1 + k \sum_{j=1}^{M} c_{nj}} \times \left(1 - \sum_{i=1}^{N} c_{im}\right) - 1\right), \quad (18)$$

where $\gamma^* = \alpha B_0/\beta$. To show that under these conditions single innervation is no longer stable, consider $N > 1$ neurons and M muscle fibres where all neurons have terminals at all muscle fibres. At equilibrium, all $dc_{nm}/dt = 0$. We deduce from eqn (18) that if at equilibrium all $c_{nm} > 0$, all $\Sigma_{j=1}^{M} c_{nj}$ and all $\Sigma_{i=1}^{N} c_{im}$ must take the same value—called u and w, respectively, where $Mw = Nu$. These constraints can be satisfied with all $c_{nm} > 0$. The value of u is such that

$$\gamma^* k \frac{a_0 - u}{1 + ku}\left(1 - \frac{N}{M}u\right) - 1 = 0, \quad (19)$$

which yields a single valid solution. Trajectories starting from points where all $c_{nm} > 0$ end on the surfaces defined by $\Sigma_{j=1}^{M} c_{nj} = \frac{M}{N} \Sigma_{i=1}^{N} c_{im} = u$. Consider an equilibrium state anywhere on these surfaces, i.e. all $dc_{nm}/dt = 0$ with all $c_{nm} > 0$ but some possibly small, and make of any number of terminals the value of c_{nm} smaller than the

equilibrium value. Then, from eqn (18), $dc_{nm}/dt > 0$ for these terminals (and others), no matter how small their values of $c_{nm} > 0$ are made, i.e. single innervation is not stable. If initially all terminals have some amount of binding complex, c_{nm}, all terminals will remain present.

The loss of stability of single innervation is in agreement with experimental findings showing that blocking nerve conduction, either during normal development or reinnervation, increases the degree of polyneuronal innervation (Thompson *et al.*, 1979; Duxson, 1982; Taxt, 1983; Ribchester, 1993).

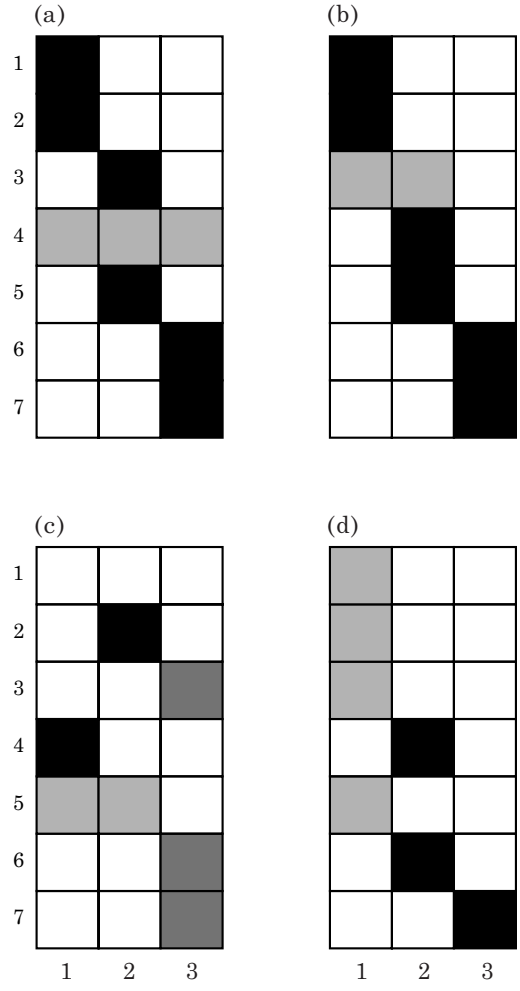

FIG. 10. Four possible stable states of the 3n7m-model. Horizontal: motor neuron number; vertical: muscle fibre number. The darkness of a square indicates the value of c_{nm} (white: $c_{nm} = 0$). Parameters: $\gamma = 400$, $k = 2$, $a_0 = 2.3$: (a) fibre 4 is polyneuronally innervated; (b) fibre 3 is polyneuronally innervated; (c) fibre 5 is polyneuronally innervated, fibre 1 is not innervated, and the other fibres are singly innervated; (d) all fibres are singly innervated.

To explain how prolonged nerve conduction block can lead to persistent polyneuronal innervation, we use the simple 2n1m-model (Fig. 11). Except for $\gamma^* k a_0 - 1 \leqslant 0$, for which there is no innervation possible at all, polyneuronal innervation is the only stable equilibrium. In normal development, starting with the initial values of binding complex c_{nm} shown in Fig. 12(a), the system goes into a state of single innervation. Note that the system is in a parameter region where there also exists a stable polyneuronal innervation equilibrium point, but normally this is not reached with low initial values of c_{nm}. Now consider development or reinnervation when electrical activity in the motor nerve is blocked, with eqn (18) governing the growth of terminals. Starting from the same initial conditions, the system goes to the only stable equilibrium that exists under these conditions: polyneuronal innervation [Fig. 12(b)]. Once the conduction block is subsequently removed, the normal equations apply again, but the starting conditions following nerve conduction block are now such that they are in the basin of attraction of the stable polyneuronal innervation equilibrium [Fig. 12(c)]. The system goes into this equilibrium and will remain there forever, i.e. persistent polyneuronal innervation. We found similar results in examples with more neurons and fibres, but polyneuronal innervation could sometimes be completely eliminated after removal of conduction block, probably as a consequence of the fact that not all configurations of polyneuronal innervation are stable (see Section 4.3).

Now consider parameter regions for which there exist no stable polyneuronal innervation equilibrium under normal conditions but only single innervation equilibria (Fig. 2 when $p < a_0 < r$ or $a_0 > s$). Again, nerve conduction block will lead to polyneuronal innervation. Removal of the block, however, will now lead to complete elimination of polyneuronal innervation as the only stable states are single innervation equilibria. This finding offers an explanation for the seemingly contradictory observations from different kinds of muscle concerning elimination of polyneuronal innervation after nerve conduction block (Brown *et al.*, 1982) by assuming that different muscles

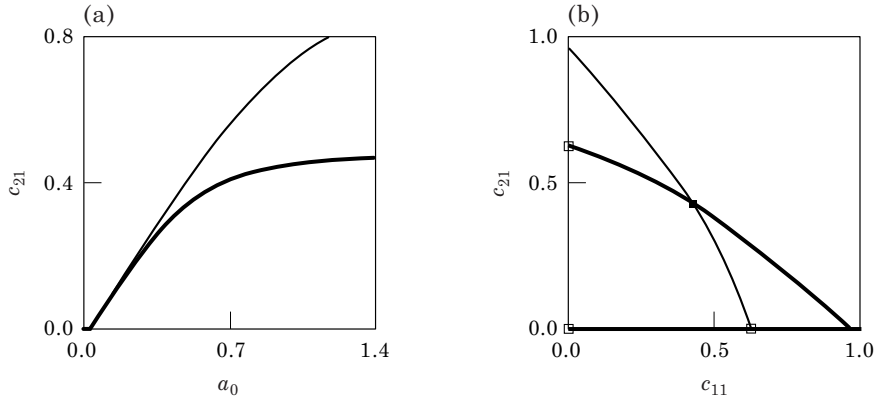

FIG. 11. The 2n1m-model under conditions of blocked electrical activity, i.e. governed by eqn (18). Under these conditions, single innervation is not stable. Except for very small a_0 where there is no innervation possible at all, polyneuronal innervation is the only stable equilibrium. In both figures, $\gamma = 17$, $k = 2$: (a) bifurcation diagram. The bold and thin lines indicate stable and unstable equilibrium points, respectively, of the whole system (i.e. $dc_{11}/dt = dc_{21}/dt = 0$) drawn on the ($a_0$, c_{21}) plane. The stable branch is the polyneuronal innervation state, the unstable one the single innervation state; (b) the bold and thin lines are the nullclines of c_{21} ($dc_{21}/dt = 0$) and c_{11} ($dc_{11}/dt = 0$), respectively; (■) indicate stable and (□) unstable equilibrium point. Parameter $a_0 = 0.8$.

have different parameter values. Some muscles will retain polyneuronal innervation following nerve conduction block, while others will completely eliminate polyneuronal innervation.

The model can also explain that other treatments than nerve conduction block can lead to persistent polyneuronal innervation (Lubishcher *et al*., 1992), by assuming that treatment changes parameter values. In Fig. 12(d) the parameter values are such that the same initial conditions as used before will lead to polyneuronal innervation. The change in parameter values has caused changes in the relative sizes of the different basins of attraction. If normal conditions are reinstalled, the starting conditions following treatment are such that they are in the basin of attraction of the stable polyneuronal innervation equilibrium [Fig. 12(e)]. The system goes to this equilibrium and will remain there forever, i.e. persistent polyneuronal innervation.

5. Conclusions and Discussion

We analysed the dual constraint model due to Bennett & Robinson (1989) for the elimination of polyneuronal innervation in developing muscle. This model combines competition for a pre-synaptic resource with competition for a post-synaptic resource to account for withdrawal of neuromuscular connections. The model has been studied before by Rasmussen & Willshaw (1993), Joseph & Willshaw (1996) and Joseph *et al*. (1997), while Van Ooyen & Willshaw (1997) showed that similar results as those described here can be produced by a more general model for the development of nerve connections.

Rasmussen & Willshaw (1993) showed that single innervation is a stable state of the dual constraint model. We have further extended the analysis of the model. Using bifurcation and phase space analysis, we showed that stable polyneuronal states and stable single innervation states can coexist. The ratio of the amount A_0 of pre-synaptic substance available per motor neuron to the amount B_0 of post-synaptic substance per endplate is an important parameter. If this ratio is not too large or too small (range depends on the other parameters) polyneuronal innervation can be stable. Other conditions for polyneuronal innervation to be stable appear to be that neurons sharing a fibre must have the same motor unit size and that neurons cannot be involved in the polyneuronal innervation of more than one fibre.

The ratio A_0/B_0 is also important in determining motor unit size (see also Rasmussen &

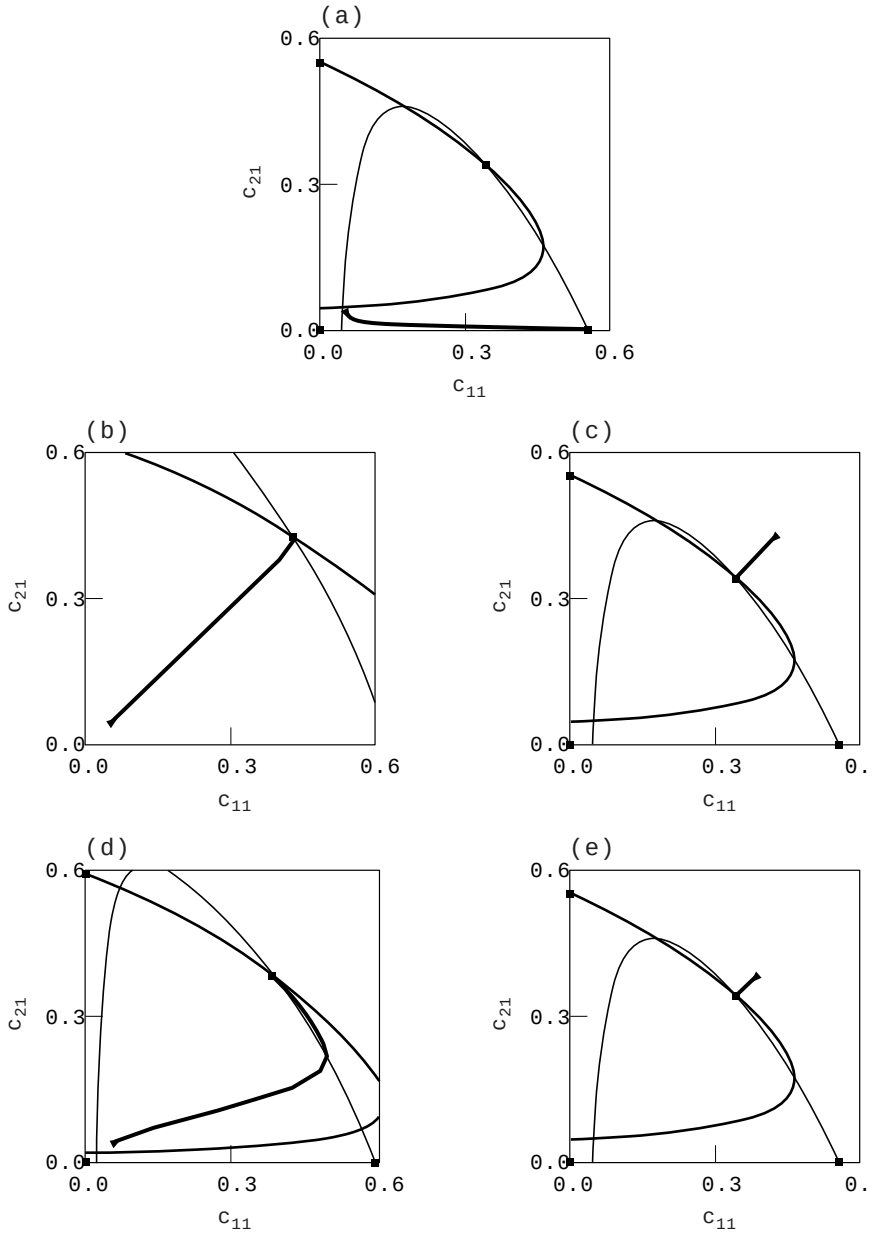

FIG. 12. Persistent polyneuronal innervation following nerve conduction block. In all figures except (d), $\gamma = 17$, $a_0 = 0.8$, $k = 2$. In (d), $k = 4$. Triangle marks starting point of a trajectory (bold line), which is ($c_{11} = 0.05$, $c_{21} = 0.04$) in (a), (b) and (d). (■) indicate stable equilibrium point; for clarity unstable equilibria are not indicated. See further text: (a) normal development, nullclines as in Fig 2(d). System goes to a single innervation state; (b) development under conditions of blocked electrical activity, nullclines as in Fig. 11(b). System goes to the polyneuronal innervation state; (c) block is removed, the nullclines are thereofre again as in (a) but starting values of c_{nm} are those reached in (b). System goes to the polyneuronal innervation state; (d) with same initial conditions as in (a) but with $k = 4$, system goes to the polyneuronal innervation state; (e) parameter k is reset to 2, but starting values of c_{nm} are those reached in (d). System goes to the polyneuronal innervation state.

Willshaw, 1993). The larger this ratio, the larger the motor unit size can be.

Which state will be reached when both polyneuronal and single innervation are stable depends on the initial values of the amounts of binding complex in the terminals, and on the sizes of the basins of attraction of the different states, which are sensitive to the values of the parameters.

The coexistence of stable polyneuronal and stable single innervation states is crucial in the explanation the model offers for results obtained

in partial denervation experiments showing that persistent polyneuronal innervation occurs after reinnervation and recovery from nerve conduction block, while normal conditions leads to elimination of most polyneuronal innervation (Brown *et al*., 1982; Barry & Ribchester, 1995). Blocking electrical activity in the model results in polyneuronal innervation, as does blocking activity in muscle during development or reinnervation (Thompson *et al*., 1979; Duxson, 1982; Taxt, 1983; Ribchester, 1993). If the parameter values are such that polyneuronal innervation states are not stable, then subsequent restoration of activity will result in withdrawal of connections until each fibre is singly innervated. However, if parameter values are such that polyneuronal innervation is also stable, then some degree of polyneuronal innervation may persist once activity has resumed. This is so because the initial amounts of binding complex in the terminals following prolonged activity block are different from those during normal development, and may lie within the basin of attraction of a state of polyneuronal innervation. Our analysis thus suggests that in order to explain the persistence of polyneuronal innervation, it is not necessary to assume that blocking activity has somehow changed the nature of the competitive interactions. Persistent polyneuronal innervation appears as a side effect of the general competitive interactions operating during normal development.

By assuming that different kinds of muscle have different parameter values, we can account for the seemingly contradictory observations that some muscles will retain polyneuronal innervation following nerve conduction block, while others will completely eliminate polyneuronal innervation (Brown *et al*., 1982).

Our analysis also suggests that it is not necessary to assume fundamentally different competitive mechanisms to explain that many muscle fibres remain polyneuronally innervated in the normal development of amphibians such as the frog (Trussell & Grinnell, 1985). Using a different set of parameter values in this case can cause normal initial amounts of binding complex to be within the basin of attraction of a state of polyneuronal innervation.

REFERENCES

Barry, J. A. & Ribchester, R. R. (1995). Persistent polyneuronal innervation in partially denervated rat muscle after reinnervation and recovery from prolonged nerve conduction block. *J. Neurosc*. **15,** 6327–6339.

Bennett, M. R. & Robinson, J. (1989). Growth and elimination of nerve terminals at synaptic sites during polyneuronal innervation of muscle cells: atrophic hypothesis. *Proc. R. Soc. Lond. B* **235,** 299–320.

Betz, W. J., Caldwell, J. H. & Ribchester, R. R. (1979). The size of motor units during post-natal development of rat lumbrical muscle. *J. Physiol*. **297,** 463–478.

Brown, M. C., Jansen, J. K. S. & van Essen, D. C. (1976). Polyneuronal innervation of skeletal muscle in new-born rats and its elimination during maturation. *J. Physiol*. **261,** 387–422.

Brown, M. C., Holland, R. L. & Hopkins, W. G. (1981). Motor nerve sprouting. *Annu. Rev. Neurosc*. **4,** 17–21.

Brown, M. C., Hopkins, W. G. & Keynes, R. J. (1982). Short- and long-term effects of paralysis on the motor innervation of two different neonatal mouse muscles. *J. Physiol*. **329,** 439–450.

De Boer, R. J. (1983). *GRIND*: *Great Integrator Differential Equations*. Bioinformatics Group, University of Utrecht, Padualaan 8, 3584 CH, Utrecht, The Netherlands.

Duxson, M. J. (1982). The effect of post-synaptic nerve block on development of the neuromuscular junction in post-natal rats. *J. Neurocytol*. **11,** 395–408.

Elliott, T. & Shadbolt, N. R. (1996). A mathematical model of activity-dependent, anatomical segregation induced by competition for neurotrophic support. *Biol. Cybern*. **75,** 463–470.

Fladby, T. & Jansen, J. K. S. (1987). Post-natal loss of synaptic terminals in the partially denervated mouse soleus muscle. *Acta Physiol. Scand*. **129,** 239–246.

Fladby, T. & Jansen, J. K. S. (1988). Selective innervation of neonatal fast and slow fibres before a net loss of synaptic terminals in mouse soleus muscle. *Acta Physiol. Scand*. **134,** 561–562.

Gouzé, J.-L., Lasry, J.-M. & Changeux, J.-P. (1983). Selective stabilization of muscle innervation during development: a mathematical model. *Biol. Cybern*. **46,** 207–215.

Grinnell, A. D. & Herrera, A. A. (1981). Specificity and plasticity of neuromuscular connections: long-term regulation of motorneuron function. *Prog. Neurobiol*. **17,** 203–282.

Jansen, J. K. S. & Fladby, T. (1990). The perinatal reorganization of the innervation of skeletal muscle in mammals. *Prog. Neurobiol*. **34,** 39–90.

Joseph, S. R. H. & Willshaw, D. J. (1996). The role of activity in synaptic competition at the neuromuscular junction. *Adv. Neural Info. Processing Syst*. **8,** 96–102.

Joseph, S. R. H., Steuber, V. & Willshaw, D. J. (1997). The dual role of calcium in synaptic plasticity of the motor endplate. In: *Computational Neuroscience*: *Trends in Research* 97 (J. Bower, ed.) pp. 7–12. New York: Plenum Press.

Lubischer, J. L., Jordan, C. L. & Arnold, A. P. (1992). Transient and permanent effects of androgen during synapse elimination in the levator ani muscle of the rat. *J. Neurobiol*. **23,** 1–9.

McArdle, J. J. (1975). Complex endplate potentials at regenerating neuromuscular junction of the rat. *Exp. Neurol.* **49,** 629–638.

Morrison-Graham, K. (1983). An anatomical and electrophysiological study of synapse elimination in the developing frog neuromuscular junction. *Devl. Biol.* **99,** 289–311.

Purves, D. & Lichtman, J. W. (1980). Elimination of synapses in the developing system. *Science* **210,** 153–157.

Rasmussen, C. E. & Willshaw, D. J. (1993). Pre-synaptic and post-synaptic competition in models for the development of neuromuscular connections. *Biol. Cyber.* **68,** 409–419.

Redfern, P. A. (1970). Neuromuscular transmission in newborn rats. *J. Neurophysiol.* **209,** 701–709.

Ribchester, R. R. (1993). Co-existence and elimination of convergent motor nerve terminals in reinnervated and paralysed adult rat skeletan muscle. *J. Physiol.* **466,** 421–441.

Taxt, T. (1983). Local and systemic effects of tetrodotoxin on the formation and elimination of synapses in reinnervated adult rat muscle. *J. Physiol.* **340,** 175–194.

Thompson, W. J. & Jansen, J. K. S. (1977). The extent of sprouting of remaining motor units in partly denervated immature and adult rat soleus muscle. *Neuroscience* **4,** 523–535.

Thompson, W. J., Kuffler, D. P. & Jansen, J. K. S. (1979). The effect of prolonged reversible block of nerve impulses on the elimination of polyneuronal innervation of new-born rat skeletal muscle fibres. *Neuroscience* **4,** 271–281.

Trussell, L. O. & Grinnell, A. D. (1985). The regulation of synaptic strength within motor units of the frog cutaneous pectoris muscle. *J. Neurosci.* **1,** 243–254.

Van Ooyen, A. & Willshaw, D. J. (1997). Competititon amongst neurons for neurotrophins. In: *Artificial Neural Networks—ICANN '97* (Gerstner, W., Germond, A., Hasler, M. & Nicoud, J.-D., eds) pp. 139–144. Berlin: Springer.

Werle, M. J. & Herrera, A. A. (1991). Elevated levels of polyneuronal innervation persist for as long as two years in reinnervated frog neuromuscular junctions. *J. Neurobiol.* **22,** 97–103.

Willshaw, D. J. (1981). The establishment and the subsequent elimination of polyneuronal innervation of developing muscle: theoretical considerations. *Proc. R. Soc. Lond. B.* **212,** 233–252.