CHAPTER 6

Dynamic mechanisms of neuronal outgrowth

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Introduction

Mature neurons are characterized by dendrites and axons with a complex and widely varying shape. These structures often exist as branched trees, which range from simple, as in pyramidal cells, to elaborate, as in Purkinje cells. The cells attain their shape during early development, after migration to their specific positions in the brain. Many studies have been and are concerned with the description of dendritic and axonal morphology, ranging from the famous studies of Ramon y Cajal (1911) to quantitative metric (Uylings, 1977; Uylings et al., 1989; Burke et al., 1992) and topological descriptions (Kleinnmann, 1987; Horsfield et al., 1989; Van Pelt et al., 1992).

In the formation of dendritic and axonal branching patterns the nerve growth cone and the cytoskeleton play a key role. The growth cone is a motile structure at the tips of outgrowing axons and dendrites, and is thought to act as the ‘eyes and ears’ of the neurite (see Letourneau et al., 1991, 1994; Kater et al., 1994). It is the structure which directly contacts the immediate environment of the neuron and which is assumed to sense other cells and gradients of soluble and membrane-bound chemical compounds. The growth cone adheres to the surrounding substrate, thereby establishing a close contact between neurite and environment. The importance of the growth cone for the development of neuritic shape lies in its involvement in guidance, elongation and branching of neurites.

The cytoskeleton is a second key factor crucial in neuritic outgrowth and maintenance of form (see Black, 1994). The cytoskeleton is a collective term for three types of molecules: intermediate filaments, actin filaments and microtubules. Intermediate filaments are the most and actin filaments the least stable molecules (Amos and Amos, 1991). Disrupting actin results in a much slower elongation rate and a lower branching probability (Marsh and Letourneau, 1984). Disrupting the microtubules can result in retraction of neurites (Yamada et al., 1970) and preventing their polymerization stops outgrowth (Bamburg et al., 1986). Among the elements of the cytoskeleton, the microtubules form a continuous core within neurites and sustain the complex neuronal shape.

What is the effect of changes in growth cone behavior or microtubule elongation on neuronal morphogenesis? And, how do these two key factors interact in neuronal outgrowth? These questions will be the subject of this chapter. Questions regarding the role of the growth cone in neuronal outgrowth will be treated in the first part of this chapter. Questions regarding the elongation of the cytoskeleton, as a limiting factor in neuronal outgrowth, are treated in the second part of this chapter. The third part aims at integrating and discussing the genesis of neuronal shape, focusing on the elongation of the neurite and...
cytoskeleton, the branched structure of neurites and the role of cellular contacts.

The growth cone

During axonal and dendritic outgrowth, the growth cone is the structure where much of the growth activity appears (see Letourneau et al., 1991, for reviews). It is the site where three kinds of actions are performed: elongation, steering and branching. The first of these depends on elongation of the cytoskeleton as well, and its discussion will be deferred to part 2 of the chapter. The latter two are mediated by the growth cone itself, in interaction with its immediate environment.

Growth cone morphology

There are two, light microscopically distinguishable domains in the growth cone, the central and the peripheral domains (Fig. 1). The central domain (or C-domain) is directly connected to the neuritic shaft, the microtubules end here like a fan and cell organelles are present. The outer part of the growth cone, the peripheral domain (or P-domain), is flattened and lacks cell organelles, and microtubules only occasionally protrude into this region (Gordon-Weeks, 1991).

The P-domain can be subdivided into two parts: the filopodia and, proximal to the filopodia, a basal part containing lamellae and veils. The lamellae and veils have a high actin content and show protrusive movements, comparable with the movements of pseudopods of amoebae (Lewis and Murray, 1992). Filopodia have an internal core of aligned actin bundles (Letourneau and Ressler, 1984; Mitchinson and Kirschner, 1988) and are very motile structures. They can be extended within minutes, have a lifespan of tens of minutes, and can be retracted again within minutes (Bray and Chapman, 1985). They adhere to the environment by forming bonds with cell adhesion molecules (CAMs), among which NCAM, N-cadherin, L1, and the integrin receptor family rank as the most important (Dodd and Jessell, 1988).

Steering of the growth cone

The filopodia are the parts of the growth cone which have to contact a substrate for guidance. Caudy and Bentley (1986) and Bentley and

Fig. 1. Morphology of the growth cone. c: central domain, p: peripheral domain, f: filopodium. Measure bar is 5 μm.
O'Connor (1991) show in the grasshopper that a filopodium has to touch a guidepost cell in order to redirect the growth cone. The distance between these guidepost cells is approximately the length of a filopodium (Ho and Goodman, 1982), allowing for a ‘stepping stone’ mode of guidance. Removing the filopodia by the addition of cytochalasin B in situ (Bentley and Toroian-Raymond, 1986) results in undirected growth by axons which would otherwise use the guidepost cells to orient themselves. Hammarback and Letourneau (1986) provided a tissue culture model of this mode of outgrowth by seeding neurons in an environment where adhesive squares were positioned within nonadhesive lanes. At small non-adhesive lane width, the neurites were able to grow over the lanes onto other adhesive squares. At a width larger than the longest filopodial length, the neurites could not cross the lanes and remained confined within their square. Filopodia and growth cones will not only adhere to substrates, but they can also be repelled by contact mediated repulsion (Kapfhammer and Raper, 1987; Schwab, 1990; Bantlow et al., 1993).

The model favored by these observations is one in which filopodia are probing the environment and transduce a signal to the growth cone, which will be received by the basal part of the P-domain. The translation from filopodial signals to growth cone displacement could be based on interference with the formation of lamellae and lamellipodia. The protrusion of lamellipodia is determined by the state of the actin cortex inside a cell or growth cone (Oster, 1988; Alt, 1990; Vallicov, 1991; Lewis and Murray, 1992; Stossel, 1993). If signals from the filopodia are able to effect the state of the actin cortex, they will be able to interfere with lamellipodial protrusions. A strong filopodial signal could cause either actin disassembly or cause a change in proteins affecting the state of the actin cortex, thereby causing lamellipodial protrusions (Alt, 1990; Lewis and Murray, 1992; Stossel, 1993). The result is that the growth cone will move in the direction of the protruding lamellipod. On the other hand, inhibitive signals (Kapfhammer and Raper, 1987; Schwab, 1990; Bantlow et al., 1993) would have an opposite effect on the actin cortex, thus causing retraction of lamellipodia.

**Branching of the growth cone**

One of the first hypotheses regarding the role of filopodia in branching was put forward by Vaughan et al. (1974), who conjectured that a branch would be formed when two filopodia simultaneously contacted synapses. This hypothesis was later generalized (Berry et al., 1980), stating that branching would occur when two filopodia simultaneously made strong adhesive bounds to the environment. Wessells and Nuttall (1978) showed that growth cones could be induced to branch when their front filopodia were lifted from the substrate, leaving only adhered filopodia at the sides. Not merely the adhesion of the filopodia, but their angular distribution was shown to be a main determinant of branching by these experiments. This idea was also put forward by Bray (1979), who attributed the increased branching of growth cones after dissecting their trailing neurite to the increased forces of sideward directed filopodia. Observations on spontaneously branching growth cones show an increased branching probability when the filopodia happen to be directed to the sides of the growth cone (Van Veen et al., 1993).

Branching is thought to be dependent not only on the angular distribution of the filopodia, but also on an interplay between filopodia and the microtubules more centrally in the neuritic shaft (Fig. 2) (Letourneau et al., 1986). Branching would occur when the filopodia orient the microtubules into distinct clusters. This configuration is attained most easily when the filopodia are oriented in distinct, sideward directed clusters. Interestingly, the addition of low doses of taxol completely prevents branching, while the filopodia are less widely spread around the central axis than in the control situation (Letourneau et al., 1987).

**A growth cone model**

To investigate the importance of filopodia in growth cone behavior, we have modeled the
growth cone as a structure existing solely of filopodia, which are present in a limited area near the basis of growth cone (Fig. 3). Both the number and directions of the filopodia are assumed to follow a stochastic distribution. These distributions are generated in the model by scattering discrete ‘adhesion sites’ on the substrate on which the modeled growth cone is positioned. Filopodial vectors are projected towards all adhesion sites within the detection area of the modeled growth cone (Fig. 3). The filopodial vectors represent the filopodia, while the detection area represents the zone around the growth cone which can be reached by filopodia. It is not proposed that in reality the number and direction of filopodia are determined by discrete adhesion sites on the substrate, but the adhesion site distribution is used to generate the stochastic distributions for the number and direction of filopodia.

The direction of growth cone displacement can be defined by the sum vector of the filopodial vectors (Fig. 3), with components:

\[ x_r = \sum_{i=1}^{n} a_i x_i \]
\[ y_r = \sum_{i=1}^{n} a_i y_i \]

where \( x_i, y_i \) are the coordinates of filopodial vector \( i \) and normalized such that \( \sqrt{x_i^2 + y_i^2} = 1 \), \( a_i \) is the weight factor of filopodial vector \( i \), \( n \) is the total number of filopodial vectors and \( x_r, y_r \) are the coordinates of the sum vector. By definition, all vectors have their origin in the basis of the growth cone and the vector \( x = 1, y = 0 \) is drawn in the direction opposite to the trailing neurite. In this approach the direction of each filopodial vector accounts for the direction, while the weight factor accounts for the strength of the filopodial action on the growth cone. A problem is that the weight factors must be determined, and presently it is difficult, if not impossible, to measure filopodial weights, either in terms of force or intracellular signals. As a first approximation, we will take each weight factor to be equal to 1, thereby focussing on the number of filopodia around the growth cone rather than the strength of the signals. In the model, the growth cone will turn left if there are more filopodia pointing to the left than to the right of the growth cone.

Experimental evidence (Wessells and Nutall, 1978; Van Veen et al., 1993) suggests that the growth cone will branch when the filopodia are
mainly directed towards the sides of the growth cone. This distribution can be quantified by the perpendicular variance \( B \) of the filopodial vectors:

\[
B = \frac{1}{n} \sum_{i=1}^{n} a_i \sin^2(\phi_i - \phi_0),
\]

where \( a_i \) and \( n \) are as defined before, \( \phi_i \) is the angle of filopodial vector \( i \) and \( \phi_0 \) is the angle of the sum vector. These angles can be calculated from the coordinates of the vectors: \( \phi_i = \arcsin(y_i) \) and \( \phi_0 = \arcsin(y_0 / \sqrt{x_0^2 + y_0^2}) \). Again, the weights of the filopodial vectors are assumed to be equal to each other. \( B \) has a value of 0 when all filopodial tips have the same direction and a value of 1 when exactly half of the number of filopodia point to each side of the growth cone.

In a homogeneous environment, the path of a tissue-cultured growth cone is a restricted random walk (Katz et al., 1984; Katz and George, 1985; Van Veen et al., 1993). This random walk of tissue cultured growth cones is mimicked to a great extent by the random walk of modeled growth cones (Van Veen et al., 1993). Van Veen and Van Pelt (1992) showed that in simulations of modeled growth cone paths the tortuosity of the path is caused by the random positions of the filopodia, and restrictions on the direction of movement imposed by the trailing neurite. In tissue-cultured growth cones, the randomness in the path is probably not caused by the random placement of the filopodia, given the lack of correlation between the angular distribution of filopodial numbers and the direction of growth cone displacement (Van Veen et al., 1993). Nevertheless, the similarity between both random walks (Van Veen et al., 1993) suggests that the random movement of tissue-cultured growth cones is also caused by random, uniformly distributed directional cues.

**The microtubule cytoskeleton**

The growth cone may be one of the principal structures in outgrowth, but the neurite formed by the growth cone must later be maintained. The main cellular components responsible for this maintenance are the cytoskeleton proteins. Of these cytoskeleton proteins, the microtubule cytoskeleton forms a rigid, continuous core within the whole neuritic tree. The microtubules are shown to be important in the maintenance of neuritic shape: disrupting the microtubules can result in retraction of neurites (Yamada et al., 1970) and preventing the polymerization of microtubules stops outgrowth (Bamburg et al., 1986). Although the microtubules cytoskeleton as such spans the whole neurite, the separate microtubules can stop and start everywhere within the neurite (Bray and Bartlett Bunge, 1981).

Microtubules have a chemical polarity which is reflected in the assembly/disassembly rates at the ends of a microtubule, these being the sites where most assembly activity takes place (Bergen and Borisy, 1980; Soifer and Mack, 1984; Amos and Amos, 1991). One end is known as the fast assembly (or +end) the other as the slow assembly (or −end). On a time scale of hours, the assembly dynamics of microtubules have been shown to be linear, with the assembly rate depending on the concentration of soluble tubulin and a constant disassembly rate (Bergen and Borisy, 1980; Soifer and Mack, 1984):

\[
\frac{1}{\tau} \frac{d \mu}{dt} = aQ - b \tag{1}
\]

where \( \mu \) is the length of the microtubule, \( \tau \) is the length of a tubulin dimer, \( Q \) is the concentration of tubulin, \( a \) is the assembly constant and \( b \) is the disassembly rate.

The assembly dynamics defined in this way suggest a steady elongation, but on a time scale of seconds, microtubules may change from an elongating to a shrinking state, a process known as dynamic instability (Mitchinson and Kirschner, 1988; Amos and Amos, 1991; Fryer et al., 1992). Dynamic instability can be fully described by the frequencies of the transitions between the elongation and the shrinkage states, and the rate constants of the elongation and shrinkage phases (Verde et al., 1992; Dogterom and Leibler, 1993).
The interplay between these four parameters determines if the average elongation rate of microtubules, as defined by Eqn. (1), will be positive, negative or zero (Dogterom and Leibler, 1993). Microtubule-associated proteins appear to increase the transition from shrinkage to elongation, thereby promoting a positive average elongation rate (Pryer et al., 1992).

There are two regions in the neurite with a high level of newly assembled parts of microtubules, namely the proximal 40 $\mu$m and the distal 100 $\mu$m (Black, 1994). It is still an open question which of these regions contributes significantly to the elongation of the neurite. Two kinds of studies suggest that assembly in the distal region is important in neurite elongation. Bamburg et al. (1986) showed that inhibition of the tubulin polymerization at the proximal part of a neurite does not affect the elongation rate, while inhibition at the distal part immediately stopped the elongation. Shaw and Bray (1977) measured the migration rate of isolated growth cones. They showed that these growth cones elongated the trailing piece of neurite with a speed leveling off in time, suggesting that assembly takes place from a pool of unassembled material near the growth cone.

The production of tubulin takes place in the soma. The half life of tubulin mRNA is only 1–2 h (Cleveland et al., 1981), ensuring that most of it will be contained in the soma because it is degraded before it is able to reach the dendrites. The spatial difference in the production and polymerization site of the tubulin protein poses a transport problem: tubulin has to be transported from the soma to the neuritic tips in order to be available for assembly. In favor of the hypothesis that tubulin is transported passively through the neurite in the form of tubulin dimers are studies which show that the concentrations profiles of a single pulse of labeled tubulin dimers (Okabe and Hirokawa, 1988; Lim et al., 1990) look very much like those of diffusing macromolecules (Popov and Poo, 1992) and also like the profiles expected from theoretical studies (Okubo, 1980; Murray, 1989). Further, experiments by Tashiro and Komiya (1992) and Hoffman et al. (1992) suggest that a large pool of soluble tubulin exists inside neurites, which is susceptible to diffusion.

It has been argued that diffusive transport is not able to carry tubulin dimers to be tips of axons over a distance in the order of millimeters (Black and Smith, 1988; Ahmad et al., 1993). However, this view is based on a solution of the diffusion equation without sources and sinks of soluble tubulin. Then, a single pulse of tubulin diffuses out forming a bell-shaped profile in space. Because no tubulin is produced, the concentration gradient levels off, and the flux of tubulin is proportional to the square root of time (Okubo, 1980). In a nerve cell, the soma forms a source of tubulin, while the assembly sites form sinks. The source and sinks keep up the gradient of soluble tubulin, which attains a constant slope and the flux of tubulin becomes constant (Van Veen and Van Pelt, 1994). In this case, diffusion is able to account for a constant transport rate of tubulin over long distances. When the drive behind tubulin transport is taken to be diffusion, the flux $J$ of material at each point between soma and tip is given by (Okubo, 1980; Murray, 1989):

$$ J = -D \frac{dQ}{dx} \quad (2) $$

where $Q$ is the concentration of soluble tubulin at position $x$ and time $t$ and $D$ is the diffusion constant.

The combined result of tubulin production, diffusive transport and polymerization, determining the elongation rate of the microtubules, has been studied by means of a mathematical model by Van Veen (Van Veen, 1993; Van Veen and Van Pelt, 1994). The model, assuming a constant tubulin production at the soma, predicts a constant elongation rate of the microtubules. This constant elongation rate is similar to observations on microtubules in tissue culture (Bergen and Borisy, 1980; Verde et al., 1992).

In the model, the tips of the neurites, being the main polymerization sites of tubulin, compete for soluble tubulin present at a common branch point (Fig. 4). Tips with a high assembly rate $a$ or a low
disassembly rate $b$ compete successfully for soluble tubulin with other tips (Figs. 4b, 4c) by creating a low tubulin concentration in the tip, and establishing a steep tubulin gradient. Differences in the tubulin diffusion constants $a$, determining the transport rate towards the tip, are of minor importance in the competition between tips (Fig. 4a).

Less successful tips not necessarily disappear because the tubulin concentrations in the soma and branch points are increasing with time (Fig. 4d). At a certain moment, less successful competitors can experience tubulin concentrations which allow them to grow. In Figs. 4b and 4c, this sequence of events is displayed, while Fig. 4d gives the tubulin concentrations accompanying Fig. 4b. Thus, competition for soluble tubulin can result in delayed outgrowth, or even retraction, of the tip with the lowest assembly or the highest disassembly rate.

The stability of neuronal microtubules increases as maturation proceeds. In highly branched dendrites, such as those of Purkinje cells, the fraction of cold stable microtubules changes from almost zero to almost one during maturation (Fairev et al., 1985). The cold stability measures the stability of microtubules by measuring their resistance to sudden cooling, which normally causes disassembly. Important factors in stabilisation are the microtubule associated proteins (MAPs) (Matus, 1988; Amos and Amos, 1991). The microtubule assembly model predicts that unstable microtubules will retract when the production of tubulin in the soma is diminished. It is proposed here that stabilization, for instance by MAPs, is essential to sustain the neuritic tree formed during outgrowth.

The genesis of neuronal shape

The growth cone and cytoskeleton determine the branching probability, the direction of elongation and the elongation rate of an emerging neuritic tree. To investigate how these actions interact in morphogenesis, the following section will focus on the elongation of segments and the formation of the branched structure. Because nerve cells seldom grow in isolation, in influence of other nerve cells on the morphogenesis will also be discussed.

Elongation

A complicating factor in relating the polymerization of the microtubule cytoskeleton to the elongation rate of the neurite is the elasticity of the neurite. Dennerl et al. (1989) and Lamoureux et al. (1992) have shown that neurites behave like simple elastics, and that they follow Hooke’s law relating force to stretching:

$$F_e = \frac{EA(t - l_0)}{l_0}$$

where $A$ is the transsectional area of the neurite, $F_e$ is the elastic force of a segment with stretched
length $l$ and unstretched length $l_0$, and $E$ is a proportionality constant. Because the growth cone exerts force on its neurite, the neurite is always in a stretched state (Lamoureux et al., 1989). As shown by Van Veen and Van Pelt (1994), the stretched length is proportional to the unstretched length, given that changes in force exerted on the neurite lead quickly to a new state of stretching. It follows also that the change in strechted length is proportional to the change in unstretched length:

$$\frac{d(l)}{dt} = k \frac{d(l_0)}{dt} \tag{3}$$

where $k = 1 + F_e/(EA)$ is a proportionality constant which is expressed in terms of the force $F_e$ that the growth cone exerts, $E$ and $A$. If the force exerted at the growth cone increases, for example by ‘towing’ the growth cone, the stretched length $l$ will increase faster in time, solely because of a larger value for $k$. This elongation effect on the elongation rate might partially explain towed growth, as demonstrated by Bray (1984) and Zheng et al. (1990). By towing, the force at the growth cone is increased, resulting in an increase in elongation rate of the stretched length.

A stretched rubber band serves as a good analogy for this type of outgrowth. Take a rubber band in both hands (grab the band somewhat from the ends, leaving these free for some distance), and stretch the band. Now, while trying to exert a constant pulling force at one hand, slip a bit of rubber band through your other hand. This results in an increase of the unstretched length of the rubber band in between your hands. Because you are trying to exert a constant force at one hand, your hands will move apart. If you slip the rubber band with a constant speed, you will notice that your hands will separate with constant speed, and the slipping speed and the separating speed are related by Eqn. (3). On top of this passive stretching effect, Buxbaum and Heidemann (1988, 1992) argue for a dependence of the assembly rate of microtubules on the amount of stretching. In their view, microtubules are compressed structures and stretching the neurite translates into a release of compression at the microtubules, giving rise to a larger assembly rate.

The interaction between tension generated by the growth cone and polymerization of the microtubules links the function of the peripheral domain, which is presumed to generate tension, to that of the central domain of the growth cone, which is presumed to generate tension, to that of the central domain of the growth cone, which is the site of tubulin assembly. This approach combines the ‘push’ and the ‘pull’ hypotheses for neuritic elongation, respectively, put forward by Bray (1987) and by Goldberg and Burmeister (1989). Bray argued that growth cones pull their neurite forward while Goldberg and Burmeister argued that the growth cone is pushed forward by material deposited in the growth cone. The mechanism put forward here postulates both a tension generating pull of the growth cone and a microtubule elongating input of tubulin into the growth cone.

**Branching**

The branched appearance of dendritic and axonal trees is highly characteristic for neurons and the name ‘tree’ refers intrinsically to a branched structure. While describing the actions of the growth cone, branching was correlated with the angular spread of the filopodia. However, this correlation gives neither information on the branch probability nor on the dependence of neuritic morphology on the branch probability.

A simplified description of the formation of dendrites and axons can be given by topological means. In this approach, the neuronal trees are treated as a collection of segments, disregarding their length, connected by branch points. At each branch point three or more segments are connected. Van Pelt and colleagues (Van Pelt and Verwer, 1986; Van Pelt et al., 1986; Verwer et al., 1992), Horsfield et al. (1987) and Kliemann (1987) developed and analyzed outgrowth models based solely on these features. Van Pelt et al. (1986) derived a model which accounts for the probability of occurrence of neuronal branching patterns, and which is attractive because of its simplicity. It
is based on only two parameters, the branch probability and an exponential function defining the dependence of the branch probability on the order of a segment. ‘Order’ is the centrifugal order, which is defined as the number of branch points between the segment and the basis of the tree. Fitting their model, Van Pelt and colleagues concluded that in rat pyramidal cells (Van Pelt et al., 1986) and rat Purkinje cells (Van Pelt et al., 1986, 1992) the branch probability decreases with increasing centrifugal order, that is towards the top of the tree, whereas multipolar non-pyramidal cells have a constant branch probability along the tree.

Van Veen and Van Pelt (1993) developed a stochastic model to study the impact of the branch probability on segment lengths within the neurite, assuming that the branch probability is constant over the whole length of the neurite. This implies that the branching probability of a piece of neurite is proportional to its length. One important constraint was incorporated: a neurite has some finite length, restricting the maximal length of a segment. With these assumptions, Van Veen and Van Pelt (1993) show that terminal segments are expected to be longer than intermediate segments, and that both types of segments decrease in length with centrifugal order. These predictions agree with measurements of rat pyramidal cells in the neocortex (Uylings et al., 1978).

Van Veen and Van Pelt (1993) show that the in vitro neurons depicted by Bray (1973) agreed nicely with the predicted results. However, the terminal segments of the in vivo basal dendrites of rat pyramidal cells were much longer than expected. This difference might well be attributed to a decreasing branch probability per unit length during development, causing terminal segments of higher order to experience a progressively lower branching probability. Burke et al. (1992) also found increasing segment lengths at smaller segment diameter in dendrites of in vivo motoneurons. Because of tapering towards the tips, thin segments generally have a high order. The branching rate is found to decrease with decreasing segment diameter and thus increasing order.

When using the measured taper and branching rates in a discrete time outgrowth model, Burke et al. (1992) reproduced their measurements, indicating that a branching probability which decreases with increasing order is an important determinant of segment lengths.

Van Veen and Van Pelt (1992) and Van Veen (1993) present simulation studies of neuritic outgrowth, where branching is determined by the angular distribution of the filopodia on the growth cone. In a homogenous environment this results essentially in a binomial branch probability per unit of length. The branch probability was shown to depend on the number of filopodia, such that the branching probability is small at very low or very high filopodial number and larger at intermediate numbers.

Forcing a constant branch probability per unit of length, the simulations yield longer terminal than intermediate segments, and decreasing segments lengths with higher order. This is exactly the pattern predicted from the stochastic model of Van Veen and Van Pelt (1993), also based on a constant branch probability per unit length. However, the simulations yield a larger topological asymmetry (expresses, on average, how much the left and right of a tree look alike at each branch point: see Van Pelt et al., 1992) and too many terminal segments in comparison with cultured and in vivo neurons. Changing the branch probability to other constant values or changing the mean number of filopodia did not change these patterns much. Instead, a decreasing branch probability with increasing centrifugal order generated more realistic values for the topological asymmetry and the number of terminal segments. These modeling results suggest that the branching pattern of neurons depends sensitively on changes in branch probability during outgrowth and that the branching probability is expected to decrease with increasing centrifugal order in real neurons.

This conclusion agrees with results from topological growth models fitted to rat pyramidal cells, where decreasing branch probabilities with increasing centrifugal order have also been found
(Van Pelt et al., 1986). Also, these topological growth models show that the expected topological asymmetry depend sensitively on an alteration of the branch probability with order.

Activity dependent outgrowth

A number of experiments using electrical stimulation (Cohan and Kater, 1986; Fields et al., 1990) or application of neurotransmitters (Haydon et al., 1987; Mattson and Kater, 1989) to an outgrowing neuron show that these treatments are able to stop or reverse the outgrowth. In tissue culture, Van Huizen and Romijn (1987) gained an increase in branching frequency after chronic blockade of bioelectric activity by tetrodotoxin in rat cerebral cells. The effect of both electrical stimulation and neurotransmitters is reversible; cessation results in a restart of outgrowth (Cohan and Kater, 1986; Fields et al., 1990). In Helisoma, a sea snail, the growth cone structure changed markedly after evoking action potentials on its trailing neurite. The number of filopodia decreased and lamellipodia retracted (Cohan and Kater, 1986; Fields et al., 1990), and, eventually, the growth cone disappeared. The effects of serotonin on neuron 19 (Haydon et al., 1987) and experimentally rising the internal calcium concentration (Rehder and Kater, 1992) are comparable with the effects of evoked action potentials.

The first steps in the cascade induced by neurotransmission are a depolarization of the membrane potential and the elevation of the internal calcium concentration (Parnas and Segel, 1980; Wadman and Connor, 1992). This elevation of the internal calcium has been made visible by calcium sensitive dyes and is observed in reaction to electrical stimulation (Kater et al., 1988), during excitatory neurotransmitter application (Wadman and Connor, 1992) and during activation by other neurons (Schilling et al., 1991). By blocking the elevation of the calcium concentration during iontophoretic application of glutamate, pyramidal cell dendrites could be prevented from retraction (Mattson and Kater, 1989), suggesting that neuritic retraction in response to neurotransmitters or electrical activity is mediated by an elevated calcium concentration.

The cascade following the elevation of calcium concentrations affects, amongst others, neuritic elongation and branching. Elevated internal calcium concentrations interfere with filopodial and vei protrusions (Goldberg et al., 1991; Rehder and Kater, 1992), probably by interfering with actin polymerization (Amos and Amos, 1991; Lewis and Murray, 1992). Calcium is also known to induce microtubule disassembly (Dustin, 1978; Gordon-Weeks, 1991; Díaz-Nido et al., 1991) at concentrations in the range of 10⁻⁶ M to 10⁻⁵ M. These concentrations are well within the physiological ranges measured in neuronal growth cones. Bantlow et al. (1993) measured a calcium concentration of 157 nM in control growth cones, while the concentration rose to 2132 nM in growth cones touching an inhibitory substrate. Al-Mohanna et al. (1992) have reported optimal neurite outgrowth at 35 nM cytosolic free calcium, while already at a calcium concentration of 60 nM a reduced neuritic outgrowth was found.

The observations indicate that calcium concentrations become elevated as soon as functional neuronal contacts are established. These elevated calcium concentrations interfere with microtubule assembly, and thus with the cytoskeleton which supports neuronal shape, explaining how neuritic outgrowth can be reduced by calcium influx resulting from neuronal contacts. In this view, the size of a neuron will be the result of a dynamic interaction with its neuronal environment. If the intensity of functional contacts changes, the morphology of the neurite will change appropriately. This principle is used by Van Ooyen and Van Pelt (1992, 1994a,b) to explain how networks of stable size are formed after an initial overshoot in neuronal connections. Neurons do change their morphology during the whole lifespan of an animal by retracting and extending branches. These morphological changes can be monitored by observing the positions of synapses (Purves et al., 1987) or by directly observing dendritic or axonal branches (Speidel, 1933; O’Rourke and Fraser, 1990).
Conclusion

During neuronal morphogenesis, branching and steering of the growth cone and microtubule elongation have been shown to be cooperating factors. While the growth cone accounts for the initiation of branch points and the stretching of neuritic segments, the tubulin dynamics determine whether branch points will survive and the elongation of the microtubule cytoskeleton supports ongoing neuritic elongation. Calcium is an important regulator of this interaction, because it changes both the functioning of the growth cone and the rate of microtubule elongation. Because calcium concentrations and electric or neurotransmitter stimulation are correlated, functional contacts are expected to change the interaction between growth cone and microtubule dynamics and, thus, to change neuronal morphology.

In this chapter, much of the interaction between the growth cone and the cytoskeleton has been explored by mathematical modeling of neuronal outgrowth. These models show that realistic neuronal outgrowth can be simulated by solely incorporating branching, steering and elongation of the growth cone and the microtubules. Of course, these models implement an hypothesis about the functioning of the growth cone and the microtubules, but the present chapter shows that there is much experimental evidence in support of the models.

References

Black, M.M. (1994) Microtubule transport and assembly cooperate to generate the microtubule array of growing axons. Chapter 4, this volume.


Speidel, C.C. (1933) Studies of living nerves. II. Activities of ameboid growth cones, sheath cells, and myelin segments, as revealed by prolonged observation of individual nerve fibers in frog tadpoles. Am. J. Anat., 52: 1–79.


