Depolarization stimulates lamellipodia formation and axonal but not dendritic branching in cultured rat cerebral cortex neurons

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Accepted 3 March 1998

Abstract

Electric activity is known to have profound effects on growth cone morphology and neurite outgrowth, but the nature of the response varies strongly between neurons derived from different species or brain areas. To establish the role of electric activity in neurite outgrowth and neuronal morphogenesis of rat cerebral cortex neurons, cultured neurons were depolarized for up to 72 h and quantitatively analyzed for changes in axonal and dendritic morphology. Depolarization with 25 mM potassium chloride induced a rapid increase in lamellipodia in almost all growth cones and along both axons and dendrites. Lamellipodia formation was dependent on an influx of extracellular calcium through L-type voltage-sensitive calcium channels. Prolonged depolarization for 24 h induced an increase in total axonal length, mainly due to an increase in branching. After three days of depolarization axonal outgrowth was largely the same as in control neurons, suggesting accommodation of the growth cones to chronic depolarization. Dendrites showed very little change during the first three days in culture, and dendritic length or branching were not affected by depolarization. Thus, in early cerebral cortex neurons depolarization specifically stimulates axonal outgrowth through increased branching. This increase in branching may be a consequence of the earlier increase in lamellipodia formation. In contrast, early dendrites seem to be unable to translate the increase in lamellipodia into changes in outgrowth or branching. This difference between axons and dendrites could be due to differences in the stabilization of the tubulin cytoskeleton.

Keywords: Neuronal morphogenesis; Growth cones; Depolarization; Neurite outgrowth and branching; Electric activity

1. Introduction

The morphogenesis of neurons is largely dependent on the activity of the growth cones at the distal endings of axons and dendrites. The growth cone serves to integrate both extracellular guidance cues and intracellular signals, and translate these into a coordinated rearrangement of the cytoskeleton [31]. In this way the growth cone mediates directed neurite outgrowth or retraction, branching and, finally, stabilization as part of a synapse onto an appropriate target cell. One of the factors which influence neurite outgrowth is electric activity [5,18,22]. The signaling mechanisms by which electric activity alters the growth cone cytoskeleton leading to changes in neurite outgrowth are not completely understood, although an influx of extracellular calcium is generally required [18,19,23,24]. The responses of neurons to depolarization, induced by electric stimulation, neurotransmitters or elevated extracellular potassium vary between neurons derived from different species or brain regions. These responses range from growth cone collapse and cessation of neurite elongation in Heliosoma neurons [5] and rat dorsal root ganglion neurons [13] to the elaboration of filopodia, lamellipodia, neurite branching and/or increased elongation rates in neuroblastoma cells [1,2] and Xenopus neurons [25]. Few studies have addressed the role of electric activity in neurite outgrowth in neurons derived from the mammalian central nervous system. A stimulation of neurite outgrowth by depolarization was mentioned in cultures of fetal rat cerebral cortex, but no details were provided [14]. In cultured rat hippocampal neurons, glutamate was found to induce a dose-dependent retardation and retraction of dendrites but not axons [23].
Since little is known about the effects of electric activity on neurite outgrowth in mammalian cerebral cortex neurons and the underlying cell biological mechanisms, we examined the effects of depolarization on neocortical neurons from fetal rats growing in culture. The effects of depolarization were investigated quantitatively along two lines: (1) short-term effects on the morphology of individual growth cones were studied with a high spatial resolution at one minute intervals, using video time-lapse microscopy, while (2) long-term effects on neuronal morphogenesis were studied after fixation of fluorescent dye-labeled neurons at 24 h and 72 h of culturing. To be able to directly compare the observations obtained with both approaches, the experiments were conducted on cultures grown under identical conditions. The cultures consisted of embryonic day 18 rat cerebral cortex neurons grown at a high density (5000 neurons/mm²) in a chemically defined medium conditioned by astrocytes [27]. To visualize the morphogenesis of individual neurons within the dense developing neuronal network, about one in 500 neurons was labeled with DiI at the time of establishing the cultures. This method yielded brightly stained single neurons against a negligible background of unstained neurons, which could easily be recorded by confocal microscopy and analyzed.

Using depolarization with 25 mM potassium chloride to mimic electrical stimulation, we observed a rapid and specific induction of lamellipodia in growth cones and along neurites. This induction was dependent on calcium influx through L-type voltage-sensitive calcium channels, and occurred at threshold intracellular calcium levels between 0.5 and 1 μM. Prolonged depolarization for 24 h increased total axonal, but not dendritic length, due to a 2-fold increase in branching. The specific induction of lamellipodia might indicate an activation of the small GTPase Rac1, which induces lamellipodia in fibroblasts and neuroblastoma cells and has been implicated in the regulation of neurite outgrowth [20,21]. As the depolarization-induced increase in axonal length and branching was preceded by increased lamellipodia formation, lamellipodia may be instrumental in neurite branching.

2. Materials and methods

2.1. Tissue culturing and fluorescent dye labeling

Culturing procedures were modified from Ref. [27]. Cerebral cortices were dissected from embryonic day 18 (E18) rats, cut into about 1 mm³ cubes and incubated with 1 ml 0.25% trypsin for 30 min in a CO₂ incubator at 36°C. After removal of most of the trypsin solution, 1 ml DMEM was added together with 200 μl soy bean trypsin inhibitor (STI) and 50 μl DNase I (500 units). After 1 min, 2 ml DMEM was added and the tissue blocks were dissociated by trituration with a Pasteur pipette. The cells were pelleted by centrifugation for 5 min at 1000 rpm. The pellet was resuspended in glia conditioned medium (GCM, see below) and a small aliquot (0.5 ml) was incubated with DiI (Molecular Probes; 50 μg/ml) for 30 min at 36°C. Meanwhile, the larger part of the cell suspension was counted and diluted to 3 million cells per milliliter in GCM. The stained cells were quickly pelleted for 10 s in an appendor centrifuge and washed three times by resuspension in 1 ml GCM containing 0.2% bovine serum albumin (BSA) and centrifugation. After the last centrifugation step, the cells were resuspended in 1 ml GCM and counted. The stained cells were diluted with the suspension of non-stained cells to obtain a ratio of 1 stained cell per 500 non-stained cells. Fifty microliters of cell suspension was pipetted into glass rings (inner diameter 7 mm; height 1.3 mm) placed on 12 mm round coverslips coated overnight with polyethylene imine (PEI, Fluka, 10 μg/ml). After the cells were allowed to adhere for 1 h in a CO₂ incubator, the rings were removed. At this stage depolarization was started by adding GCM diluted with depolarization stock solution (consisting of 150 mM potassium chloride plus 2 mM calcium chloride and 2 mM magnesium sulfate), to obtain a final concentration of 25 mM K⁺ in the culture medium. Control cultures received GCM diluted in the same way with control stock solution (147 mM sodium chloride, 3 mM potassium chloride, 2 mM calcium chloride and 2 mM magnesium sulfate) to compensate for the dilution of the GCM. Cultures were maintained at 36°C in a CO₂ incubator at 100% humidity.

Cultures which were used in time-lapse experiments were established as described above, without the additional staining of part of the cells. After centrifugation, the cells were resuspended in 1 ml DMEM, and treated subsequently with trypsin (200 μl 0.25%, 1 min), STI (0.25%) and DNase I (500 units, 1 min), to remove most of the dead cells and cell debris. The suspension was adjusted to 10 ml with DMEM, centrifuged, resuspended in 10 ml GCM and counted. Fifty microliters of cell suspension (150,000 cells) was plated into glass rings placed eccentrically on 30 mm coverslips coated with PEI. After 1 h the rings were removed and 100 μl GCM containing 0.2% BSA was added. The cultures were maintained in a CO₂ incubator until use.

GCM was produced by incubating primary glial cultures for four days with a chemically defined medium consisting of 75% DMEM and 25% Ham’s F12, from which glutamate and aspartate were deleted and to which were added: insulin (10 μg/ml); human apo-transferrin (50 μg/ml), thiotic acid (0.25 μg/ml), tocopherol (10 μg/ml), retinol (1 μg/ml), biotin (0.1 μg/ml), sodium pyruvate (100 μg/ml), glutamine (30 μg/ml; all from Sigma) and penicillin/streptomycin. GCM contained 2 mM calcium chloride and 2 mM magnesium sulfate [27]. The glial cultures (consisting mainly of astrocytes) were established from postnatal day one to three rat cerebra,
dissociated by trituration and seeded at a density of one brain (two cerebria) per 175 cm² tissue culture flask in 50 ml D10 medium, consisting of DMEM with 10% heat-inactivated fetal calf serum and penicillin/streptomycin (all media from Gibco).

2.2. Analysis of changes in growth cone morphology

Coverslips (30 mm diameter) containing cerebral cortex cultures aged one to seven days in vitro were quickly mounted in an incubation chamber and incubated in 1 ml culture medium at 36°C. The culture was then clamped into the temperature-controlled stage of an Axiovert 135TV inverted microscope (Zeiss). The culture was kept at 36°C and constantly gassed with 5% CO₂. The temperature around the heated stage was stabilized by a perspex cage surrounding most of the microscope except the oculars, the phototube and the light sources. After 30 min acclimatization, time-lapse recording was started using a VIDAS25 image analyzer (Kontron, Munich, Germany) which was programmed to open a light shutter for 5 sec every minute and to record an image at a resolution of 256 × 256 pixels. One recording session typically lasted 30 min. In most cases, stimulation was started 10 min after time lapse recording was begun. Growth cones were imaged with a Zeiss plan neofluar lens (40 × , n.a. 1.30) and an intermediate obtovar lens (2.5 × ) using phase contrast optics. The image was recorded with a three-colour CCD camera (KY-F55, JVC) of which the green channel was fed into the VIDAS image analyzer.

Cultures were stimulated by bath application of depolarizing, control media or reagents by taking out 0.5 ml medium from the incubation chamber, mixing this with the stimulation medium and then gently returning the medium to the incubation chamber at t = 0 min. To depolarize or give a control stimulus, 0.2 ml depolarization or control stock solution (see Section 2.1) was added to the cultures, respectively. Pharmaca were usually dissolved in dimethyl sulfoxide (DMSO) as 1000 × concentrated stock solutions. DMSO at 0.1% did not affect growth cone morphology. In the calcium clamping experiment, extracellular free calcium was clamped at 0.1, 0.5, 1 and 10 μM by adding 2 mM BAPTA and 0.506, 1.26, 1.55, 1.95 mM calcium chloride, respectively, to calcium-free culture medium without phenol red. Medium with 0.1 mM free calcium contained 0.1 mM calcium chloride without BAPTA.

Growth cones were analyzed using an IBAS2000 image analyzer by tracing the outer circumference (excluding the filopodia) and indicating the filopodia as vectors starting at the growth cone boundary and ending at the distal tip of the growth cone. From this the growth cone area and number and length of filopodia were calculated. The border between the growth cone and the neurite shaft was defined in growth cones before stimulation as the region where the neurite showed a clear broadening. Depolarization in many cases induced an apparent shortening or disappearance of filopodial vectors due to partial or complete engulfment by lamellipodia without a retraction of the distal tip. Based on the assumption that the proximal tip of the filopodia was not influenced by stimulation, but merely engulfed, a corrected filopodia length was computed, taking the pre-stimulus proximal end and the post-stimulus distal tip to construct the vector.

2.3. Analysis of neuronal morphology

Following one to three days of treatment, cultures containing DiI stained neurons were fixed overnight at 4°C in 4% paraformaldehyde and 0.3% glutaraldehyde in phosphate buffered saline (PBS: 10 mM sodium phosphate buffer, pH 7.3 in 150 mM sodium chloride). After several rinses in PBS the cultures were mounted in Mowiol embedding medium and stored at 4°C in the dark. DiI stained neurons containing at least one neurite were recorded using a Zeiss 410 confocal microscope with a 40 × (n.a. 1.3) lens (zoom 1) and stored as tif files at a resolution of 1024 × 1024 pixels. The tif files were analyzed in a sequence of four consecutive programs using an IBAS2000 image analyzer (Kontron). In the first program, running in automatic mode, the soma was identified and the neurites were skeletonized to lines with a width of one pixel. In the second program, the skeletonized images were compared with the original images and, if needed, edited manually to match the original image. In the third program, running in automatic mode, the different 'substructures' were identified. These included the soma, the axon, the different dendrites emerging from the soma, and per neurite the branch points, intermediate and terminal segments and filopodia. The axon was defined as the longest neurite.
Fig. 2. Time-lapse series of a single growth cone, showing the effects of depolarization with 25 mM potassium chloride. Acquisition was started at 1-min intervals at $t = -10$ min until 10 min after depolarization. Depolarization was started at $t = 0$ min. The insert shows the size of the growth cone area as a function of time (in minutes).
according to Dotti et al. [10]. The distinction between filopodia and (very short) neurites or terminal segments was defined by length. Extensions longer than 0.7 μm and shorter than 5.1 μm were designated filopodia. Extensions equal to or longer than 5.1 μm were designated neurites if they emerged from the soma, or terminal segments if one end of the extension was attached to a neurite. The cut-off was based on visual inspection of a large number of recorded neurons, which indicated 5 μm to be an optimal value. Very few filopodia-like structures were longer than 5 μm, while very few ‘real’ neurites (which were usually more complex and thicker than filopodia) were found below 10 μm. The automatic identification of the neuronal substructures was verified interactively and, if necessary, adjusted. In the final program, the values of the different parameters were calculated. The following parameters were determined for the cell body (Fig. 1): the soma area, the number of neurites and the number of filopodia emerging from the soma. For the axon were measured: the total length without filopodia, the number of branch points (which equals the number of intermediate segments; both are equal to the number of terminal segments = the number of endpoints minus 1), the number of filopodia per axon (expressed per micrometer), the mean intermediate and terminal segment length, and the mean ‘radial distance’ (the length of the straight vector from the origin of the axon to the different endpoints). For the dendrites, the same parameters were measured as for the axons. Since none of the parameters showed a normal distribution, groups were statistically compared using the nonparametric Mann–Whitney test.

3. Results

3.1. Effects of depolarization on growth cone morphology

Depolarization with 25 mM potassium chloride induced in most growth cones a rapid increase in surface area (49 out of 54; 91%), due mainly to the expansion of lamellipodia (Fig. 2). Out of 36 growth cones which received a control stimulus, three showed a depolarization-like response (8%). The earliest changes observed occurred at about 1.5 min, with an average time of onset of 2.25 min (±0.1 min, S.E.M.; Table 1). In many cases, changes in growth cone morphology ceased after 4 min (average 5.28 ± 0.27 min). No further changes were observed up to 20 min after stimulation. Lamellipodia formation was not confined to the growth cones, but was also prominent along neurites. Filopodia were occasionally lost, but this seemed almost exclusively due to engulfment by lamellipodia (Fig. 3). In a few cases two filopodia were seen to merge in a zipper-like fashion within a few seconds.

In cases where the quality of the images was good enough (10 depolarized and 16 control growth cones), several growth cone parameters were quantitated at 1 min before and 5 min after stimulation (Table 1). Depolarization increased the total growth cone area by almost 40%, while no change was observed upon control stimulation. As this change was due to the extension of lamellipodia, which make up only part of the total growth cone area (together with the thicker central area), the relative increase in lamellipodia was probably several-fold larger. The number of filopodia was decreased upon depolarization, but this was solely due to engulfment by lamellipodia. The average length of the remaining filopodia decreased by about 20%, but after correction for the shift in growth cone boundary (‘engulfment’), filopodia length was reduced only 5% by depolarization. Under control conditions filopodial number or length were not altered. Thus, depolarization specifically increased lamellipodial area without affecting filopodia. The increase in lamellipodial area remained as long as the neurons were depolarized (tested up to 20 min). In two cases we tested whether the response was reversible when the potassium concentration was returned to 3 mM. In both cases the lamellipodia were retracted and could again be induced by a second depolarizing stimulus. To check whether the induction of lamellipodia could be due to changes in osmolarity rather than depolarization, the response of growth cones to both hypotonic (+20%) and hypo-osmotic (−20%) stimulation was recorded. Neither of these conditions resulted in the induction of lamellipodia in growth cones or along the neurites. However, in both cases filopodia were retracted.

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 16)</th>
<th>Depolarization (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth cone area before stimulus</td>
<td>7928 ± 1051</td>
<td>7463 ± 1006</td>
</tr>
<tr>
<td>Increase in area</td>
<td>−1.6% ± 1.9%</td>
<td>37.2% ± 10.7%</td>
</tr>
<tr>
<td>Number of filopodia before stimulus</td>
<td>11.0 ± 1.1</td>
<td>14.0 ± 1.5</td>
</tr>
<tr>
<td>Increase in filopodia number</td>
<td>−0.10 ± 0.10</td>
<td>−3.65 ± 1.3</td>
</tr>
<tr>
<td>Filopodia length before stimulus</td>
<td>45.1 ± 4.9</td>
<td>45.3 ± 3.4</td>
</tr>
<tr>
<td>Increase in filopodia length</td>
<td>−0.74 ± 0.55</td>
<td>−9.5 ± 2.0</td>
</tr>
<tr>
<td>Increase in filopodia length after correction*</td>
<td>−0.56 ± 0.53</td>
<td>−2.7 ± 1.1</td>
</tr>
</tbody>
</table>

*The length of filopodia which were not lost was corrected for ‘engulfment’ by lamellipodia as described in Section 2.
Fig. 3. Depolarization-induced changes in growth cone area (a) and filopodia length (b,c) as a function of time. Growth cone area increased mainly between one and two minutes after depolarization. When not corrected for engulfment, several filopodia decrease in length and even disappear. When corrected for engulfment by lamellipodia, filopodia length does not change, indicating that filopodia are not retracting.

3.2. The role of calcium in depolarization-induced lamellipodia formation

To test the role of calcium in the depolarization-induced formation of lamellipodia, cultures were pretreated for 30 min with various reagents which interfere with calcium mobilization, followed by stimulation with 25 mM potassium chloride or a control stimulus. The responses were recorded from 10 min before until 20 min after stimulation (Table 2). When extracellular calcium was omitted and remaining calcium chelated with EGTA (1 mM) none of the growth cones tested formed lamellipodia after depolarization or a control stimulus. Blocking calcium entry through L-type voltage sensitive calcium channels with nifedipine prevented lamellipodia induction in all eight growth cones tested (Table 2). Chelation of extracellular calcium or nifedipine treatment by themselves caused a gradual loss of lamellipodia and a thinning of filopodia, indicating that basal calcium entry is required for maintaining growth cone shape. Chelation of intracellular calcium with the membrane penetrable agent BAPTA-AM prevented lamellipodia formation following depolarization. These observations indicate that the induction of lamellipodia formation by depolarization is dependent on an influx of extracellular calcium through L-type calcium channels. Since many of the cellular responses mediated by calcium are dependent on binding to and activation of calmodulin [15], we tested whether blocking calmodulin would prevent the response of growth cones to depolarization. Using the potent and specific calmodulin antagonist calmidazolium (0.1 μM; IC₅₀ 0.5 μM [30]) lamellipodia formation in response to depolarization was suppressed in six out of eight growth cones, while in two growth cones lamellipodia were formed with a similar timing as in the absence of calmidazolium.

Finally, we determined the threshold level at which intracellular calcium induced lamellipodia formation. This was done by ‘clamping’ free extracellular calcium at a fixed concentration using the calcium chelator BAPTA, after which 1 μM ionomycin was added to allow calcium entry into the neurons. At 1 μM free calcium all four growth cones tested generated lamellipodia at two to three minutes, while at higher concentrations lamellipodia were induced within 30 s (Table 3). At 0.1 and 0.5 μM free calcium, no lamellipodia were formed, except for one out of three growth cones tested at 0.1 μM.

3.3. Effects of depolarization on neuronal morphogenesis

To determine the long-term effects of depolarization on neuronal morphogenesis, cerebral cortex neurons were depolarized with 25 mM potassium chloride, starting at 1 h after plating. Cultures were fixed at 24 h and 72 h. Dil labeling was found to stain mainly individual cells, revealing even filopodia with excellent contrast and negligible background staining (Fig. 4). Virtually all stained cells showed a neuronal morphology, characterized by a clearly rounded cell body and thin extensions, which were branched to a variable degree. At the earliest time point in our study (24 h) almost all neurons had one neurite which was clearly longer (about 4-fold on average; Fig. 6b,c) than the other neurites. This neurite was taken to be the axon, based on the observations of Dotti et al. [10] in
### Table 2

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>Depolarization</th>
</tr>
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<tbody>
<tr>
<td>No antagonist</td>
<td>8% (3/36)</td>
<td>91% (49/54)</td>
</tr>
<tr>
<td>Calcium-free medium</td>
<td>0% (0/2)</td>
<td>0% (0/2)</td>
</tr>
<tr>
<td>Nifedipine (10 μM)</td>
<td>0% (0/1)</td>
<td>0% (0/8)</td>
</tr>
<tr>
<td>BAPTA-AM (10 μM)</td>
<td>0% (0/3)</td>
<td>0% (0/3)</td>
</tr>
<tr>
<td>Calmidazolium (0.1 μM)</td>
<td>0% (0/4)</td>
<td>25% (2/8)</td>
</tr>
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</table>

The cultures were acclimatized in medium containing antagonist for 30 min after which they were depolarized with 25 mM potassium chloride or received a control treatment. Induction of lamellipodia was rated by comparing growth cones at 1 min before and 5 min after stimulation. Calcium-free medium consisted of culture medium from which calcium was omitted and to which 1 mM EGTA was added. BAPTA-AM induced lamellipodia formation on its own, but no further stimulation was seen upon depolarization.

### Table 3

<table>
<thead>
<tr>
<th>[Free Ca$^{2+}$]</th>
<th>Proportion of growth cones showing lamellipodia induction</th>
<th>Start of response</th>
</tr>
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<tbody>
<tr>
<td>0.1 μM</td>
<td>33% (1/3)</td>
<td>0.5 min</td>
</tr>
<tr>
<td>0.5 μM</td>
<td>0% (0/3)</td>
<td>—</td>
</tr>
<tr>
<td>1.0 μM</td>
<td>100% (4/4)</td>
<td>2–3 min</td>
</tr>
<tr>
<td>10 μM</td>
<td>100% (3/3)</td>
<td>0.5 min</td>
</tr>
<tr>
<td>100 μM</td>
<td>100% (2/2)</td>
<td>0.5 min</td>
</tr>
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</table>

The cultures were acclimatized in medium containing a fixed concentration of free extracellular calcium after which calcium entry was induced with 1 mM ionomycin as described in Section 2.

Hippocampal cultures, that by 12 h in vitro, one of the neurites has begun to elongate rapidly and becomes the axon if left unperturbed [11]. Sometimes clusters of two or more labeled cells were observed. Since it was difficult to unequivocally distinguish which processes belonged to which cell body, clustered cells were excluded from analysis. Cells without extensions or with broad extensions or a ‘fibroblast morphology’ (which constituted less than a few percent of the stained cells) were also omitted from analysis.

Qualitative observations suggested that upon depolarization neurons showed longer and more branched neurites (axons) and a higher density of filopodia along the neurites (Fig. 5). This was further analyzed quantitatively.

#### 3.3.1. ‘Cell body’ parameters

The surface area of the cell body increased from about 95 μm$^2$ at 24 h to 140 μm$^2$ at 72 h in culture (Fig. 6a). Depolarization resulted in a striking increase in cell surface area by about 60% at 24 h, which normalized by 72 h. The number of neurites emerging from the cell body showed a small (statistically not significant) decrease between one and three days, and was not affected by depolarization (Fig. 6d).

#### 3.3.2. Axons

Total axonal length showed a significant increase between one and three days under control conditions and was stimulated by depolarization on day one, but less at three days (Fig. 6b). These changes in total length were accompanied by similar increases in the number of axonal branching points with time in culture and following depolarization (Fig. 6e). The average intermediate segment length increased between one and three days in culture, but was not significantly affected by depolarization (Fig. 6h). Terminal segment length did not increase between one and three days in vitro, but was decreased after 24 h of depolarization (Fig. 6k). Taken together, these observations indicate that the primary effect of depolarization was to increase axonal branching rather than accelerate growth cone propagation. The average radial distance followed the same trend as total axonal length; it increased between one and three days and was significantly increased by depolarization at day one but not at day three (data not shown). The density of filopodia along axons increased between one and three days in depolarized, but not in control cultures. Depolarization did not significantly affect the density of filopodia on day one, but after three days the density was 2-fold higher level than in control cultures (Fig. 6g). Average filopodia length measured between 3 and 3.5 μm and was not affected by age or treatment (data not shown).

#### 3.3.3. Dendrites

Total dendrite length showed a non-significant increase of only 35% between one and three days in culture and was not affected by depolarization (Fig. 6c). Dendritic growth cones showed a similar increase in density of filopodia between one and three days in depolarized cultures (Fig. 6j).
branching was very limited during the first three days in culture: on average, only one in two dendrites showed a branch point. In fact, the majority of dendrites (70%) did not show any branching, while a small subpopulation (11%) showed multiple branching. Neither age nor depolarization affected dendritic branching (Fig. 6f). The mean intermediate segment length per dendrite (based on the small population of dendrites which showed branching) was significantly increased by depolarization at day one, but not thereafter (Fig. 6i). However, this increase in mean intermediate segment length at day one was offset by a 20% decrease in dendritic branching. As a result, depolarization had no effect on total dendrite length. Terminal segment length was not affected by depolarization or age in culture (Fig. 6l). The average radial distance increased between one and three days, but was not significantly
Fig. 6. Induction of changes in neuronal morphogenesis by depolarization with 25 mM potassium chloride for 24 h or 72 h. Displayed are the mean values ± S.E.M. Statistical comparison between the depolarized and control group of the same age, using the Mann–Whitney test: *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. Statistical comparison between similar treatment groups at one and three days in culture: †p ≤ 0.05; ‡p ≤ 0.01; §§p ≤ 0.001. Numbers of neurons or axons in the different groups: day 1 control: n = 17; day 1 depolarization: n = 33; day 3 control: n = 24; day 3 depolarization: n = 29. Numbers of dendrites in the different groups: day 1 control: n = 62; day 1 depolarization: n = 127; day 3 control: n = 71; day 3 depolarization: n = 110.

affected by depolarization (data not shown). The density of filopodia along dendrites decreased almost 50% between one and three days in culture (Fig. 6j). Depolarization induced a 3-fold increase in filopodial density on day one and an almost 2-fold increase on day three in culture (Fig. 6j). Filopodia along dendrites showed a similar length as along axons (2.9–3.3 μm). Filopodia length was not influenced by age or depolarization (data not shown).
4. Discussion

4.1. Depolarization-induced changes in lamellipodia formation

The first noticeable effect of depolarization on neuronal morphology was the rapid induction of lamellipodia within about 2 min. Pharmacological evidence showed that this response required an influx of extracellular calcium through L-type calcium channels. Depolarization with elevated potassium also induced calcium-dependent lamellipodia formation in differentiated N1E-115 neuroblastoma cells [1], while direct current (DC) electric field stimulation caused similar effects in N1E-115 cells [2], *Xenopus* neurons [25], and rat hippocampal dendrites [8]. In contrast, in *Heliosoma* neurons [6] and rat dorsal root ganglion neurons [13] depolarization induced collapse of growth cones and neurite retraction. Thus, the response of growth cones to depolarization, although apparently always calcium-dependent, is dependent on the type of neuron. The response may even depend on the axonal or dendritic origin of the growth cone [8]. We did not see any differences between axons and dendrites. In fact, lamellipodia formation was not restricted to the growth cone, but also occurred along the shafts of axons and dendrites.

The differential responsiveness of neurons to depolarization may be explained by differences in optimal calcium levels required for maintaining growth cone structure and neurite outgrowth. As proposed by Kater et al. [18], growth cones are optimally maintained at a specific intracellular calcium level: at low calcium levels, growth cones collapse and neurites may be arrested or retract; at (very) high calcium levels, possibly toxic effects may induce growth cone collapse and neurite retraction. Since depolarization stimulates lamellipodia formation and neurite outgrowth in cerebral cortex neurons, it would appear that (at least during the first week in culture) resting calcium levels are below the optimum, and that depolarization brings calcium levels closer to the optimum. We found a threshold for the induction of lamellipodia between 0.5 and 1 μM intracellular free calcium, with a similar time course as with depolarization. This threshold was close to the range of 0.2 to 1 μM intracellular calcium observed in fast moving growth cones of rat diencephalon, as compared to levels from 30 to 200 nM in stalled or slowly moving growth cones [7]. Rapid increases in intracellular calcium ranging from 0.5 to 1.5 μM have been reported in response to depolarization with 40 to 50 mM KCl (which we found to be equipotent to 25 mM KCl in inducing lamellipodia) in neurons cultured from various brain regions [3,6,33]. When calcium levels of 10 μM or higher were introduced into the neurons, growth cones showed a very rapid swelling and neurons died within minutes, presumably due to the toxic effects of high intracellular calcium concentrations (reviewed by Choi [4]). Taken together, our observations are in agreement with the calcium hypothesis [18], but further experiments are needed to more precisely determine the threshold levels of intracellular calcium for inducing lamellipodia and modulating neurite outgrowth. An influx of calcium through L-type calcium channels was clearly also important under non-stimulated conditions, since both extracellular calcium chelation and nifedipine induced a gradual loss of lamellipodia. Preliminary observations indicate that in the long run, nifedipine reduces neurite outgrowth (Lequin and Ramakers, unpublished observations).

Since many calcium-dependent responses are mediated via binding to calmodulin [15], its involvement in lamellipodia induction was investigated using the calmodulin antagonist calmidazolium. Calmidazolium, at a low concentration [30], suppressed lamellipodia formation in 75% of the growth cones, thus implicating calmodulin in the induction of lamellipodia by depolarization. Although calmodulin is involved in the activation of many enzymes, a likely candidate in this case would be myosin light chain kinase [16].

Although we did not investigate other signaling factors, the specific induction of lamellipodia, i.e., without effects on filopodia, could point to the involvement of Rac1, a member of the Rho-family of small GTPases. In fibroblasts Rac1 specifically induces lamellipodia by regulating the polymerization and rearrangement of the actin cytoskeleton, whereas filopodia formation is dependent on activation of cdc42 [26]. Introduction of activated Rac1 into neuroblastoma cells induces large lamellipodial growth cones and stimulates neurite extension [32]. We propose that depolarization-induced lamellipodia formation results from an influx of extracellular calcium which upon binding to calmodulin activates a signaling cascade involving Ras [29] and Rac [28], leading to rearrangement and/or de novo polymerization of actin filaments. The rapid onset of lamellipodia induction provides a nicely delimited time-frame of 2 min for unraveling the sequence of signaling events by which depolarization induces changes in growth cone morphology.

4.2. Depolarization-induced changes in neurite outgrowth

Prolonged depolarization of cerebral cortex neurons for one or more days induced a large increase in cell body size after 24 h, but not thereafter. This increase probably reflected a stimulation of cell metabolism, which normalized during the next two days. Depolarization stimulated total axonal length at 24 h, largely due to a doubling in axonal branching. In contrast, total dendrite length was not affected by depolarization. Dendrites also did not show any changes in total length or branching between one and three days, when axonal length and branching were more than doubled. A similar development occurs in hippocampal neurons, where dendrites stop elongation for several days after their initial formation, while axons keep growing [10]. Thus, dendritic elongation may be actively sup-
pressed during the first few days in culture, or prevented by the lack of an essential factor. The same mechanism could be responsible for the absence of changes in dendrite length or branching upon depolarization. Dendrites were not incapable of responding, however, since depolarization elicited dendritic lamellipodia during the first minutes and later increased the density of filopodia. Apparently, however, these changes did not translate into permanent changes in dendrite morphology. Lamellipodia and filopodia formation depend upon actin filaments, whereas neurite elongation is largely dependent on microtubules [31]. Thus, in dendrites the transition from more dynamic actin-based structures to more stable tubulin-based dendritic branches appears to be blocked for several days after the initial minor dendrites are formed. In contrast, in axons changes in growth cones and lateral filopodia do result in increased outgrowth and branching. This idea is supported by observations in early hippocampal neurons. In these neurons most axonal branches arise from lateral filopodia, while at the same time dendritic filopodia show motility, but do not contribute to branch formation or net elongation [10]. Further growth of dendrites may require expression of microtubule-stabilizing factors, such as microtubule-associated proteins.

An increase in neurite outgrowth after depolarization has been described before in cultured rat cerebral cortex neurons [14]. However, this study dealt mainly with the stimulation of neuronal survival by depolarization, and did not provide any details on neurite outgrowth [14]. Neuronal cell death was very low in our cultures during the first week, and did not seem to be affected by depolarization. This was probably due to the fact that we cultured neurons at a much higher density and in an optimized chemically defined medium conditioned by astrocytes, two conditions we know to be highly beneficial to neuronal survival in culture [27]. In cultured Xenopus neurons, DC stimulation promoted neurite branching [25], while in neuroblastoma cells depolarization stimulated neurite elongation [1]. In contrast, in cultured Helix neurons [6] and rat dorsal root ganglion cells [13] electric stimulation induced an arrest of neurite elongation and even retraction. Generalizing, stimulation of neurite outgrowth is usually correlated with the induction of large growth cones, often with abundant lamellipodia, while growth cone arrest or retraction is preceded by the loss of filopodia and lamellipodia. Thus, the induction of lamellipodia along axons may be a first step in the generation of collateral branches. Since we studied growth cones for up to 20 min after stimulation, we do not know whether depolarization-induced lamellipodia formation is a transient phenomenon. In rat dorsal root ganglion neurons growth cone collapse induced by electrical stimulation reversed within 24 h despite continued stimulation [13]. This reversal was presumed to be due to accommodation of the growth cones to the increased calcium influx [12]. Since most of the changes induced by depolarization at 24 h were lost by three days, accommodation did seem to occur, but only after several days. Prolonged time-lapse observations up to 24 h after depolarization should establish whether lamellipodia serve a specific role in neurite elongation and/or branching, comparable to the specialized function proposed for filopodia as the sensors of growth cones [9,17].

Acknowledgements

We gratefully acknowledge the assistance of G. van der Meulen in reproducing the photographs and Prof. M.A. Corner for his editorial comments.

References


