The Journal of Neuroscience, November 1991, 11(11): 3371–3378

Distribution of Ca^{2+} and Na^{+} Conductances during Neuronal Differentiation of Chick DRG Precursor Cells

K. Gottmann,[1] H. Rohrer,[2] and H. D. Lux[1]

[1]Department of Neurophysiology and [2]Department of Neurochemistry, Max Planck Institute for Psychiatry, D-8033 Planegg-Martinsried, Germany

The distribution of Ca^{2+} and Na^{+} conductances on neuronal precursor cells was investigated during differentiation. Ionic conductances on the soma or on the growth cone were isolated by superfusing all other parts of the cells with sucrose. Conductances on the neuritic shaft were detected as additional conductances after removing sucrose from the neuritic shaft. Neuronal precursor cells were isolated from chick dorsal root ganglia by selectively killing differentiated neurons.

Cultured precursor cells differentiated into morphological and functional mature neurons. Functionally undifferentiated precursor cells (during the first 10 hr in culture) expressed only low-voltage–activated (LVA) Ca^{2+} currents. High-voltage–activated (HVA) Ca^{2+} and Na^{+} currents appeared delayed after more than 10 hr in culture.

Voltage-dependent conductances, if expressed by a cell, were present on all parts of the surface membrane at all stages of differentiation. LVA Ca^{2+} conductances were well represented on the growth cone as well as on the soma in functionally undifferentiated precursor cells. During differentiation of precursor cells, LVA Ca^{2+} and HVA Ca^{2+} as well as Na^{+} conductances were expressed on the somatic membrane, on the neuritic shaft, and on the growth cone. These results demonstrate the expression of Ca^{2+} channels on growth cones during differentiation.

Growth cones are specialized structures controlling neurite growth. Evidence for Na^{+} channels on growth cones (O'Lague et al., 1985; Gottmann and Lux, 1990) has been obtained. Voltage-dependent Ca^{2+} channels in the growth cone have been demonstrated in a variety of preparations (Anglister et al., 1982; Bolsover and Spector, 1986; Cohan et al., 1987; Streit and Lux, 1987; Haydon and Man-Son-Hing, 1988; Lipscombe et al., 1988; Gottmann and Lux, 1990). A quantitative analysis of the distribution of Ca^{2+} conductances during sprouting of pheochromocytoma (PC12) cells revealed high densities of Ca^{2+} channels on the growth cone and on the somatic membrane, whereas Ca^{2+} channel density was extremely low along the neurites (Streit and Lux, 1989). Growth cone Ca^{2+} channels seem to be organized in clusters forming local hot spots (Lipscombe et al., 1988; Silver et al., 1990; Streit and Lux, 1990).

Received Feb. 12, 1991; revised May 31, 1991; accepted June 7, 1991.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 220 A1,A8). We thank C. Krieger and V. Bulka for excellent technical assistance.

Correspondence should be addressed to K. Gottmann, Max Planck Institute for Psychiatry, Department of Neurophysiology, Am Klopferspitz 18A, D-8033 Planegg-Martinsried, Germany.

In vertebrate neurons, two types of Ca^{2+} conductances are well distinguished on the basis of their voltage dependence of activation: high-voltage–activated (HVA; N- and L-type) and low-voltage–activated (LVA; T-type) Ca^{2+} channels (Carbone and Lux, 1987; Tsien et al., 1988). In contrast to HVA Ca^{2+} channels, LVA Ca^{2+} channels had been suggested to be restricted to the somatic membrane (Llinas and Yarom, 1981; Yaari et al., 1987; Haydon and Man-Son-Hing, 1988) but have recently been shown in growth cones of regenerating dorsal root ganglion (DRG) cells (Gottmann and Lux, 1990). Activation of growth cone Ca^{2+} channels by action potentials seems to mediate inhibition of neurite outgrowth by increasing intracellular Ca^{2+} (Kater et al., 1988; Silver et al., 1989; Fields et al., 1990). By contrast, increased intracellular Ca^{2+} seems to promote increases in the growth cone area (Anglister et al., 1982; Goldberg, 1988; Silver et al., 1989, 1990). These seemingly contradictory roles of intracellular Ca^{2+} might indicate differential sensitivity of neurite elongation and growth cone motility. Growth cone Ca^{2+} channels also may play a role in transmitter release from growth cones (Hume et al., 1983; Young and Poo, 1983).

Studies on the distribution of voltage-dependent channels have so far been conducted only on differentiated or regenerating neurons, but not during neuronal differentiation. Isolated neuronal precursor cells from chick DRG have been shown to differentiate in culture to neurons (Rohrer et al., 1985). The time course of appearance of Ca^{2+} and Na^{+} conductances has been described in detail previously (Gottmann et al., 1988). Now we report the distribution of Ca^{2+} and Na^{+} channels in differentiated neurons, in precursor cells during the appearance of Ca^{2+} and Na^{+} conductances, and in functionally undifferentiated precursor cells.

Materials and Methods

Neuronal precursor cells from chick DRG were isolated and cultured as described previously (Rohrer et al., 1985; Gottmann et al., 1988). Briefly, DRG from embryonic day 6 (E6) chick embryos were dissociated to single cells. Differentiated neurons and glial cells were selectively killed by treatment with monoclonal antibodies Q211 and O4 in the presence of complement. Killing efficiency was over 95% (Rohrer et al., 1985). Isolated precursor cells were cultured in F14 medium supplemented with 10% horse serum, 5% fetal calf serum, and 20 ng/ml NGF.

Conductances on the membrane area of interest were isolated after blocking the conductances on other parts by superfusion with isotonic sucrose (in mM): 250 sucrose, 20 HEPES, pH 7.3, adjusted with CsOH. Briefly, a laminar sucrose stream was created with an inflow pipette, containing sucrose, and an outflow pipette. Using the sharp boundary zone between sucrose and the Na^{+}/Ca^{2+}-containing bath solution (<20 μm; Streit and Lux, 1989; Gottmann and Lux, 1990), parts of a cell were superfused with sucrose. The boundary zone was visible under phase-contrast optics. The sucrose-superfused membrane areas were

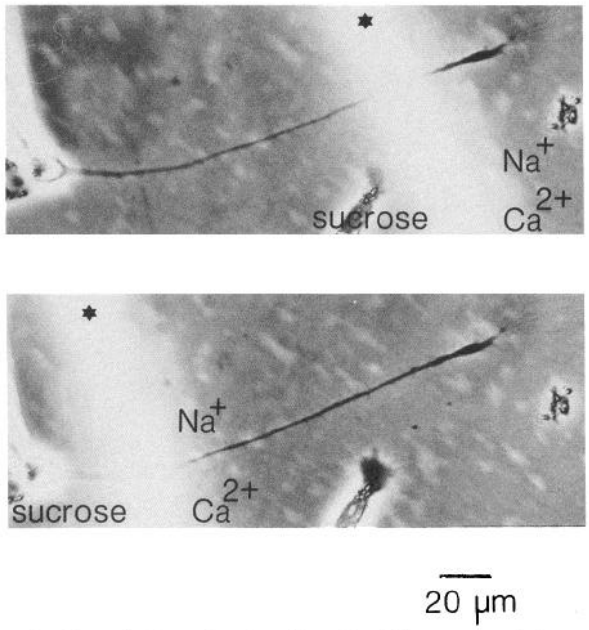

Figure 1. Superfusion of parts of a cell with sucrose. *A*, Soma and neuritic shaft were in sucrose, while the growth cone was kept in Na^+/Ca^{2+}-containing solution. The boundary zone (*) between sucrose and Na^+/Ca^{2+} solution was visible with phase-contrast optics. Isolated growth cone currents were recorded by a somatic patch pipette. *B*, After repositioning of the inflow pipette, only the soma was in sucrose. Conductances located on the neuritic shaft were now unblocked.

varied by repositioning of the inflow pipette during recording from the same cell (Fig. 1). Superfusion of freshly dissociated DRG neurons with sucrose completely blocked voltage-dependent inward currents (data not shown).

Recording conditions and data analysis were as described previously (Carbone and Lux, 1987; Gottmann et al., 1988; Gottmann and Lux, 1990). Whole-cell currents were recorded using the patch-clamp technique (Hamill et al., 1981). Patch pipettes were located on the cell soma. The spatial separation of recording electrode and voltage-dependent conductances limits this method to neurites with small enough electrotonic distances. Estimations of electrotonic voltage decay in PC12 neurites showed an attenuation to 95% of the initial value at the end of neurites 2 μm in diameter and 150 μm in length (Streit and Lux, 1989). Therefore, cells that had grown out only one neurite not exceeding 200 μm were selected. Growth cone currents are attenuated in amplitude at the somatic recording site by less than 10% along a 150 μm neurite 2 μm in diameter (Streit and Lux, 1989).

The internal solution contained (in mM) 100 CsCl, 20 tetraethylammonium (TEA)–Cl, 10 glucose, 0.25 $CaCl_2$, 10 EGTA, 5 Mg-ATP, 0.25 cAMP, and 10 HEPES (pH 7.3). Resistance of patch pipettes was 5–7 MΩ. The following external solutions were used: (1) to record inward currents (in mM): 130 NaCl, 10 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, pH 7.3; (2) to isolate Na^+ currents (in mM): 110 NaCl, 10 $MgCl_2$, 20 HEPES, pH 7.3; and (3) to isolate Ca^{2+} currents (in mM): 110 choline Cl, 10 $CaCl_2$, 20 HEPES, pH 7.3.

The discrimination between the two principal types of Ca^{2+} currents (Carbone and Lux, 1987; Tsien et al., 1988) was based on the differences in voltage dependence. The properties of LVA and HVA Ca^{2+} currents in precursor cells are identical to more differentiated DRG neurons (Gottmann et al., 1988). LVA Ca^{2+} currents activate between −60 and −50 mV membrane potential and reach maximal current amplitude between −30 and −20 mV. In contrast, HVA Ca^{2+} currents start to activate at −20 mV and reach maximal amplitude at 0 to +10 mV. Therefore, at −30 mV only LVA Ca^{2+} conductances contribute to the Ca^{2+} current. LVA Ca^{2+} currents are selectively blocked by 50–100 μM Ni^{2+}. This treatment reduced the amplitude of Ca^{2+} currents at +10 mV membrane potential by less than 10% in chick DRG neurons (Carbone et al., 1987; Gottmann and Lux, 1990). This demonstrates that at +10 mV Ca^{2+} currents are almost exclusively HVA Ca^{2+} currents; only a minor contamination with LVA Ca^{2+} currents is present. Therefore, the Ca^{2+} current at +10 mV is referred to as HVA Ca^{2+} current. Additionally, HVA Ca^{2+} currents show a much slower inactivation than LVA Ca^{2+} currents (Carbone and Lux, 1987; Gottmann et al., 1988).

Results

Distribution of Na^+ and Ca^{2+} conductances in differentiated neurons

Neuronal precursor cells isolated from chick DRG (E6) differentiate *in vitro* into neurons. After more than 25 hr in culture,

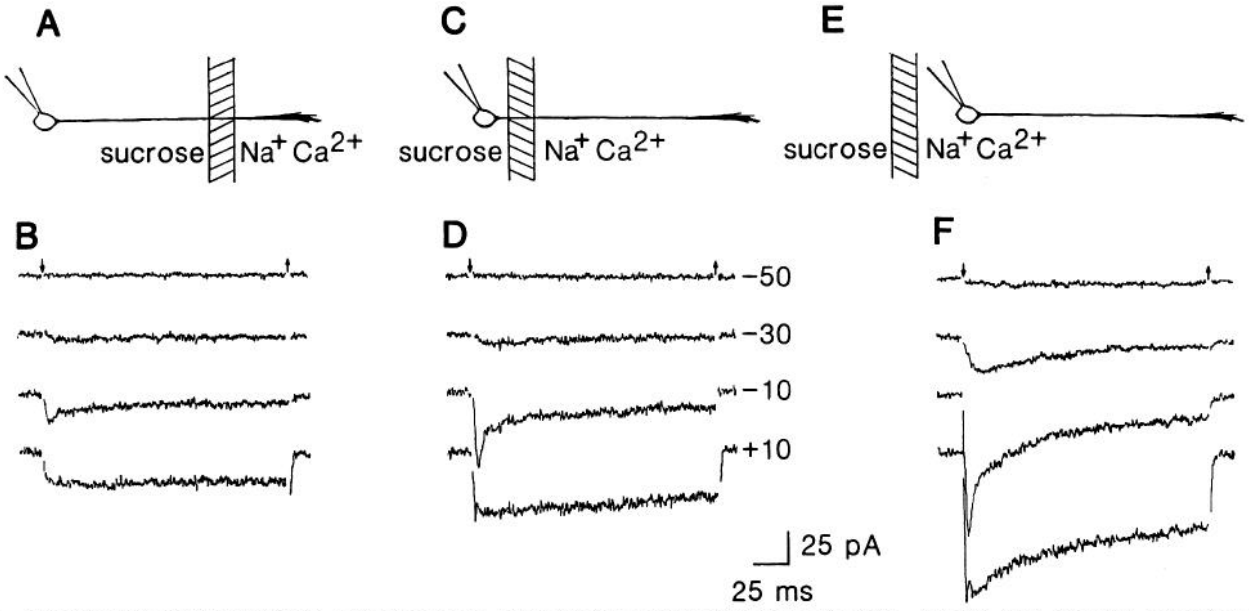

Figure 2. Distribution of Na^+ and Ca^{2+} conductances in differentiated neurons. Holding potential, −80 mV. Cell was step depolarized to the indicated membrane potentials. *A*, *C*, and *E*, Sketches of recording conditions. The sucrose-superfused part of the cell was varied. Bath contained 130 mM Na^+ and 10 mM Ca^{2+}. The boundary zone between sucrose and Na^+/Ca^{2+}-containing solution is illustrated as *hatched area*. *B*, *D*, and *F*, Corresponding current records. Na^+ and Ca^{2+} conductances were found on the growth cone (*B*). Additional conductances appeared after unblocking of channels on the neuritic shaft (*D*) and the soma (*F*).

they show typical neuronal morphology, are characterized by neuron-specific markers (Rohrer et al., 1985), and express voltage-dependent Na^+, LVA, and HVA Ca^{2+} currents (Gottmann et al., 1988). Neurons that had grown out one neurite of about 150 μm in length with a growth cone area of about 30 μm were selected (Fig. 1). K^+ currents were blocked by exchanging intracellular K^+ with Cs^+ and intracellular application of TEA.

Growth cone conductances were isolated by superfusing the soma and the neuritic shaft with sucrose. The growth cone was kept in a Na^+/Ca^{2+}-containing solution (Fig. 2*A*). Inward currents were elicited in 11 out of 12 growth cones by 200 msec step depolarizations from a holding potential of −80 mV (Fig. 2*B*). After superfusing the whole cell with sucrose, growth cone currents were completely blocked ($n = 3$).

To examine Na^+ and Ca^{2+} conductances on the neuritic shaft and on the somatic membrane, soma and neuritic shaft were first superfused with sucrose and growth cone currents were recorded. Then, only the soma was superfused with sucrose and the neuritic membrane, in addition to the growth cone, was exposed to Na^+/Ca^{2+}-containing solution (Fig. 2*C*). Conductances on the neuritic shaft could now contribute to the current. An increase in the amplitude of inward currents was observed in seven out of seven cells (Fig. 2*D*). In the final step, growth cone, neurite, and soma were exposed to Na^+/Ca^{2+}-containing solution (Fig. 2*E*). A further increase in the amplitude of inward currents was seen in all cells ($n = 7$) (Fig. 2*F*).

Thus, voltage-dependent conductances (Na^+, Ca^{2+}) were expressed on the somatic and the neuritic membranes, as well as on the growth cone membrane.

Glial precursor cells, identified by morphological criteria (phase-dark cell bodies, no neurites), after more than 25 hr in culture did not show inward currents (Na^+, Ca^{2+}) comparable in amplitude to those of neurons (less than 10% of the size of the neuronal currents in all cells).

Distribution of Ca^{2+} conductances during differentiation of precursor cells

During the first 10 hr in culture, precursor cells express only LVA Ca^{2+} currents. HVA Ca^{2+} currents appear after 10 hr, and after more than 25 hr all neurons show both Ca^{2+} currents. Between 10 and 25 hr, some of the cells did not yet express HVA Ca^{2+} currents (Gottmann et al., 1988).

The distribution of Ca^{2+} conductances was studied in precursor cells cultured for 10–25 hr. Ca^{2+} currents were isolated using a Na^+-free, Ca^{2+}-containing (10 mM) extracellular solution. Cells were held at −80 mV and were step depolarized to various membrane potentials for 200 msec.

Isolated somatic Ca^{2+} currents were recorded after the peripheral conductances had been blocked by superfusing the neuritic shaft and the growth cone with sucrose. Then, the whole cell was exposed to Ca^{2+}-containing solution (Fig. 3*A*).

LVA as well as HVA Ca^{2+} conductances were expressed on the somatic membrane in 8 out of 12 cells. The mean amplitude of somatic LVA Ca^{2+} currents at −30 mV membrane potential (see Materials and Methods) was 20.7 ± 15.6 pA; the mean amplitude of somatic HVA Ca^{2+} currents at +10 mV membrane potential was 54.3 ± 38.2 pA. After exposing the whole cell to Ca^{2+}-containing solution, LVA (−30 mV) as well as HVA (+10 mV) Ca^{2+} current amplitudes increased (Fig. 3*B*). Mean whole-cell LVA Ca^{2+} current amplitude was 31.9 ± 21.5 pA, and mean whole-cell HVA Ca^{2+} current amplitude was 85.8 ± 54.9 pA. Comparison of the *I/V* characteristics obtained in both recording situations demonstrated that Ca^{2+} current amplitude increased over the whole range of potentials and that no shifts in the *I/V* characteristic occurred (Fig. 3*C*). The increase in HVA current amplitude was observed in all eight cells; the increase in LVA current amplitude was observed in six of eight cells. In the remaining two cells, no change in LVA current amplitude was seen. The ratio of somatic to whole cell Ca^{2+} current amplitudes was similar for both LVA (0.72 ± 0.25 pA at −30 mV) and HVA (0.63 ± 0.07 at +10 mV) Ca^{2+} currents (Fig. 3*D*).

Only a part of the precursor population cultured for 10–25 hr expressed HVA Ca^{2+} currents (Gottmann et al., 1988). In fact, 4 out of 12 cells showed only LVA Ca^{2+} currents, suggesting ongoing differentiation. In these cells, Ca^{2+} current amplitude was maximal at −20 mV membrane potential and Ca^{2+} currents inactivated completely within 100 msec at +10 mV membrane

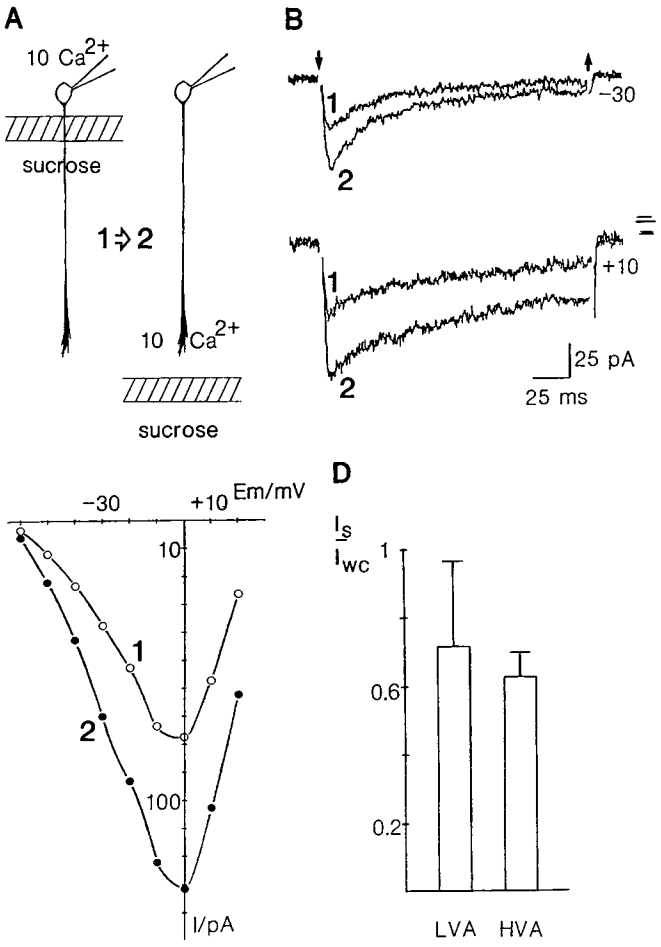

Figure 3. Distribution of Ca^{2+} conductances in differentiating precursor cells. *A*, First, peripheral conductances were blocked with sucrose, while soma was in 10 mM Ca^{2+} (*1*). After unblocking of channels on neurite and growth cone, the additional conductances contributed to the current (*2*). *B*, Current records. Holding potential, −80 mV. Depolarizing pulses elicited LVA (*−30*) as well as HVA (*+10*) Ca^{2+} currents during recording from the soma (*1*). After unblocking of conductances on the peripheral membrane (*2*), LVA (*−30*) as well as HVA (*+10*) current amplitudes increased. *Arrows* indicate beginning and ending of depolarizing pulse. *C*, Corresponding *I/V* relationships demonstrating LVA and HVA conductances on both the soma (*1*) and the peripheral membrane. *2*, whole cell. *Em*, membrane potential. *D*, Ratios of somatic Ca^{2+} current amplitude (I_s) to whole-cell Ca^{2+} current amplitude (I_{wc}). Peak amplitude at −30 mV (*LVA*) and at +10 mV (*HVA*) was used. *Error bars* are SD.

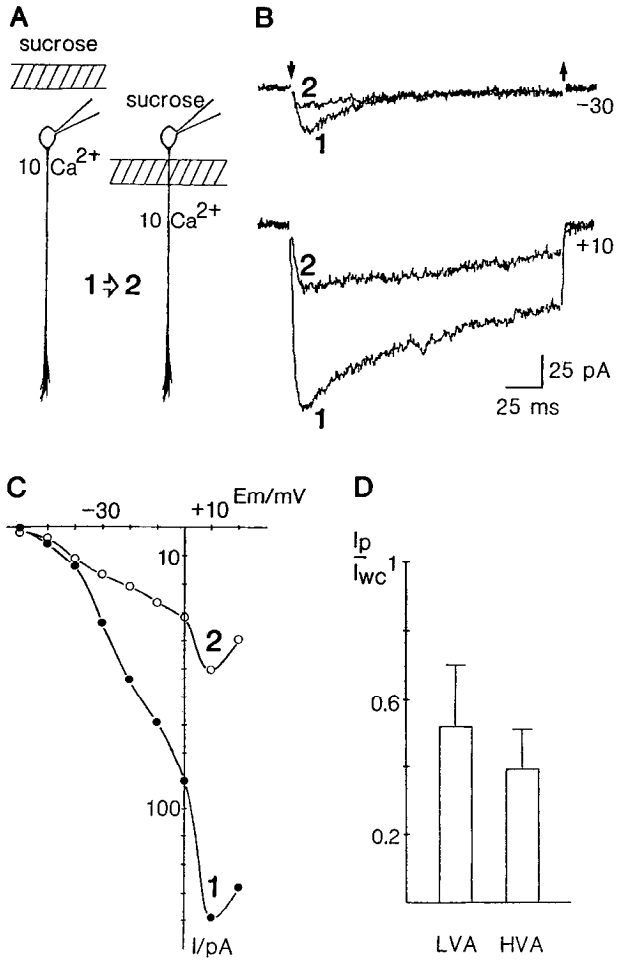

Figure 4. Isolation of peripheral Ca^{2+} conductances. *A*, First, the whole cell was exposed to 10 mM Ca^{2+} (*1*), and then somatic conductances were blocked with sucrose (*2*). *B*, Current records. Holding potential, −80 mV. After blocking of somatic conductances, whole-cell LVA (*−30*) as well as HVA (*+10*) Ca^{2+} currents (*1*) decreased in amplitude (*2*), but both types of Ca^{2+} conductances were present on peripheral membrane areas. *Arrows* indicate beginning and end of depolarizing pulse. *C*, Corresponding *I/V* relationships. *1*, whole cell; *2*, peripheral membrane. *Em*, membrane potential. *D*, Ratios of peripheral (I_p) to whole-cell Ca^{2+} current amplitudes (I_{wc}) *Error bars* are SD.

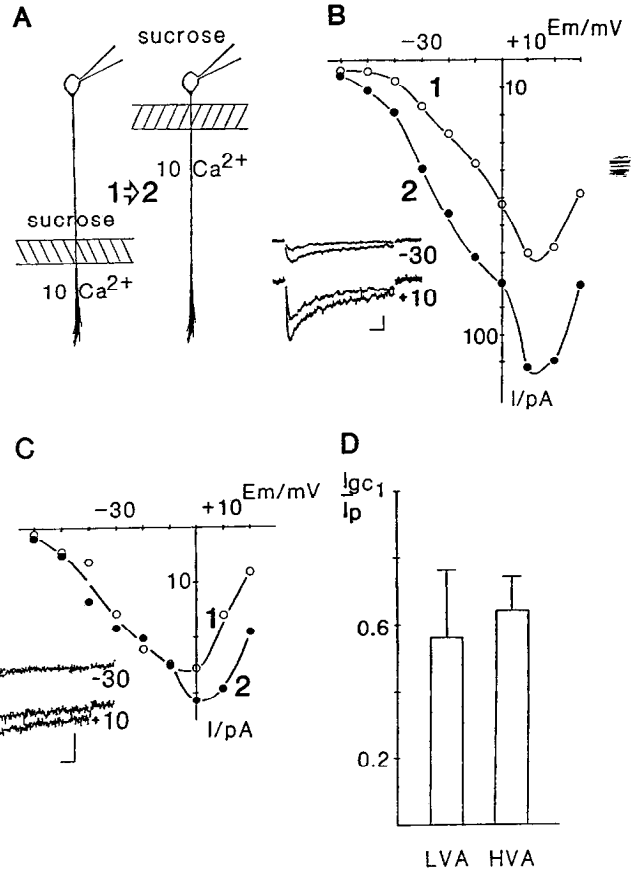

Figure 5. Distribution of peripheral Ca^{2+} conductances. *A*, First, growth cone conductances were isolated by superfusing soma and neuritic shaft with sucrose (*1*). Then, conductances on the neuritic shaft were unblocked (*2*). *B* and *C*, *I/V* relationships from two representative cells. *Insets* illustrate current records. Calibration, 25 pA and 25 msec. Holding potential, −80 mV. Growth cone (*1*) LVA as well as HVA currents were present. After unblocking of channels on the neuritic shaft (*2*), either LVA and HVA Ca^{2+} current amplitudes (*B*) or only HVA current amplitude increased (*C*). *Em*, membrane potential. *D*, Ratios of growth cone (I_{gc}) to peripheral Ca^{2+} current amplitudes (I_p). *Error bars* are SD.

potential. After unblocking of peripheral Ca^{2+} conductances, LVA Ca^{2+} currents increased.

To confirm the presence of peripheral Ca^{2+} conductances, they were demonstrated in isolation. Whole-cell Ca^{2+} currents were recorded first, and then the somatic conductances were blocked by superfusion with sucrose (Fig. 4*A*). All five cells investigated expressed LVA and HVA Ca^{2+} currents. Mean whole-cell LVA Ca^{2+} current amplitude was 34.6 ± 8.0 pA (−30 mV); mean whole-cell HVA Ca^{2+} current amplitude was 94.3 ± 30.0 pA (+10 mV). After isolation of peripheral Ca^{2+} conductances, both types of Ca^{2+} currents were clearly resolved in all cells (Fig. 4*B,C*). Mean peripheral LVA Ca^{2+} current amplitude was 18.0 ± 7.7 pA; mean peripheral HVA Ca^{2+} current amplitude was 35.2 ± 10.6 pA. The ratio of peripheral to whole-cell Ca^{2+} current amplitudes was similar for both LVA (0.52 ± 0.18) and HVA (0.39 ± 0.12) currents (Fig. 4*D*).

In summary, HVA Ca^{2+} conductances were expressed on the somatic as well as on the peripheral membrane. Similarly, LVA Ca^{2+} conductances were present on the somatic as well as on the peripheral membrane. Also, the quantitative distribution of both Ca^{2+} conductances was comparable.

To study peripheral Ca^{2+} channels further, growth cone conductances were isolated by superfusing soma and neuritic shaft with sucrose. Then, the neuritic shaft, in addition to the growth cone, was exposed to Ca^{2+}-containing solution (Fig. 5*A*). LVA as well as HVA Ca^{2+} conductances were expressed on the growth cone membrane in 8 out of 10 cells. Mean growth cone LVA Ca^{2+} current amplitude was 9.2 ± 5.2 pA (−30 mV); mean growth cone HVA Ca^{2+} current amplitude was 36.2 ± 19.4 pA (+10 mV). After exposing the neuritic shaft and the growth cone to Ca^{2+}-containing solution, HVA Ca^{2+} current amplitude increased in all cells (Fig. 5*B,C*). LVA Ca^{2+} current amplitude increased in five cells (Fig. 5*B*), whereas in three cells no increase occurred (Fig. 5*C*). Mean peripheral LVA Ca^{2+} current amplitude was 18.3 ± 13.3 pA; mean HVA Ca^{2+} current amplitude was 59.0 ± 33.4 pA. The ratio of growth cone to peripheral Ca^{2+} current amplitude was similar for LVA (0.56 ± 0.20) and HVA (0.64 ± 0.11) Ca^{2+} currents.

Two out of 10 cells did not yet express HVA Ca^{2+} currents at all. In both cells, growth cone LVA Ca^{2+} currents were ob-

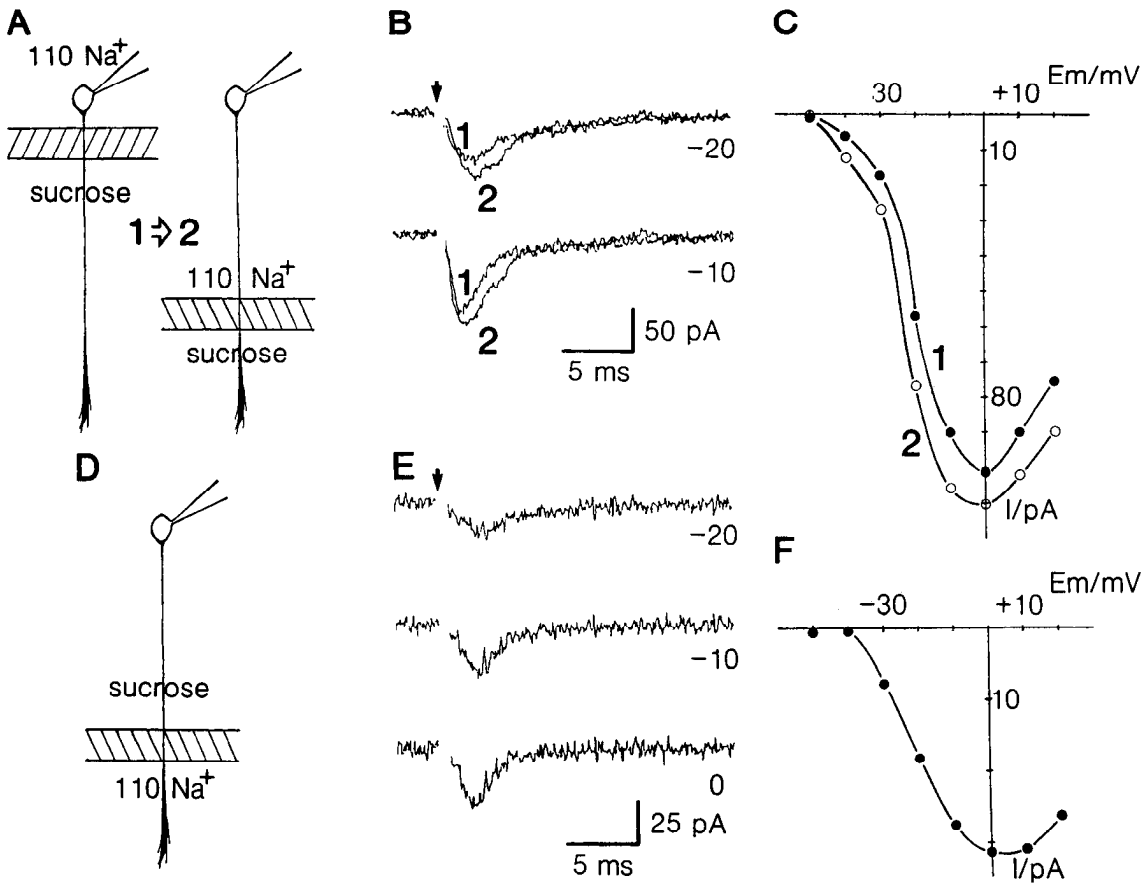

Figure 6. Distribution of Na^+ conductances in differentiating precursor cells. *A*, Soma was in 110 mM Na^+; peripheral conductances were blocked with sucrose (*1*). Then, conductances on the neuritic shaft were unblocked (*2*). *B* and *C*, Corresponding current records (*B*) and *I/V* relationships (*C*). Holding potential, −80 mV. Na^+ conductances were present on the soma (*1*). After unblocking of channels on the neuritic shaft, Na^+ current amplitudes increased (*2*). *Em*, membrane potential. *Arrows* indicate beginning and end of depolarizing pulses. *D*, To isolate growth cone Na^+ conductances, soma and neuritic shaft were superfused with sucrose. *E*, Growth cone Na^+ currents. *F*, Corresponding *I/V* relationship.

served and LVA Ca^{2+} currents increased in amplitude after unblocking of neuritic Ca^{2+} conductances.

In summary, precursor cells expressed HVA Ca^{2+} conductances on the growth cone membrane and on the neuritic shaft. Similarly, LVA Ca^{2+} conductances were represented on the growth cone as well as on the neuritic shaft. However, in several cells LVA Ca^{2+} channels were not found on the neuritic shaft (Table 1).

Distribution of Na^+ conductances during differentiation of precursor cells

During the first 10 hr in culture, DRG precursor cells do not exhibit voltage-dependent Na^+ currents. Simultaneously with the HVA Ca^{2+} current, Na^+ currents start to be expressed after 10 hr in culture, and after more than 25 hr all cells show Na^+ currents (Gottmann et al., 1988).

The distribution of Na^+ conductances in precursor cells was determined after 10–25 hr in culture. To isolate Na^+ currents, Ca^{2+} was replaced by Mg^{2+} in the Na^+ (110 mM)-containing extracellular solution. Cells were held at −80 mV and were step depolarized to various membrane potentials for 50 msec.

First, somatic Na^+ currents were recorded in isolation after superfusing the growth cone and the neuritic shaft with sucrose. Then, Na^+ conductances on the neuritic shaft were unblocked (Fig. 6*A*). Somatic Na^+ currents were expressed in five out of seven cells. Mean somatic current amplitude at 0 mV membrane potential was 186.7 ± 141.7 pA. After exposing the neuritic shaft to Na^+-containing solution, the amplitude of Na^+ currents increased in all five cells over the whole range of potentials (Fig. 6*B,C*); mean current amplitude at 0 mV was 229.2 ± 168.0 pA. The remaining two cells did not yet express Na^+ currents in any part of the cell.

To study Na^+ conductances in the growth cone, soma and neuritic shaft were superfused with sucrose and the growth cone was kept in Na^+-containing solution (Fig. 6*D*). Growth cone Na^+ currents were clearly resolved in five out of nine cells (Fig. 6*E,F*). Mean amplitude was 18.3 ± 11.7 pA at 0 mV. In the remaining four cells, again Na^+ currents were not expressed in any part of the cell.

In summary, Na^+ conductances were similar to Ca^{2+} conductances expressed on the somatic, neuritic, and growth cone membranes (Table 1).

Distribution of Ca^{2+} conductances in functionally undifferentiated precursor cells

Functionally undifferentiated precursor cells (during the first 10 hr in culture) exhibit only LVA Ca^{2+} currents. Na^+ and HVA Ca^{2+} currents are not yet expressed (Gottmann et al., 1988). In the present investigation, LVA Ca^{2+} currents were recorded in a Ca^{2+} (10 mM)- and Na^+-containing solution to demonstrate

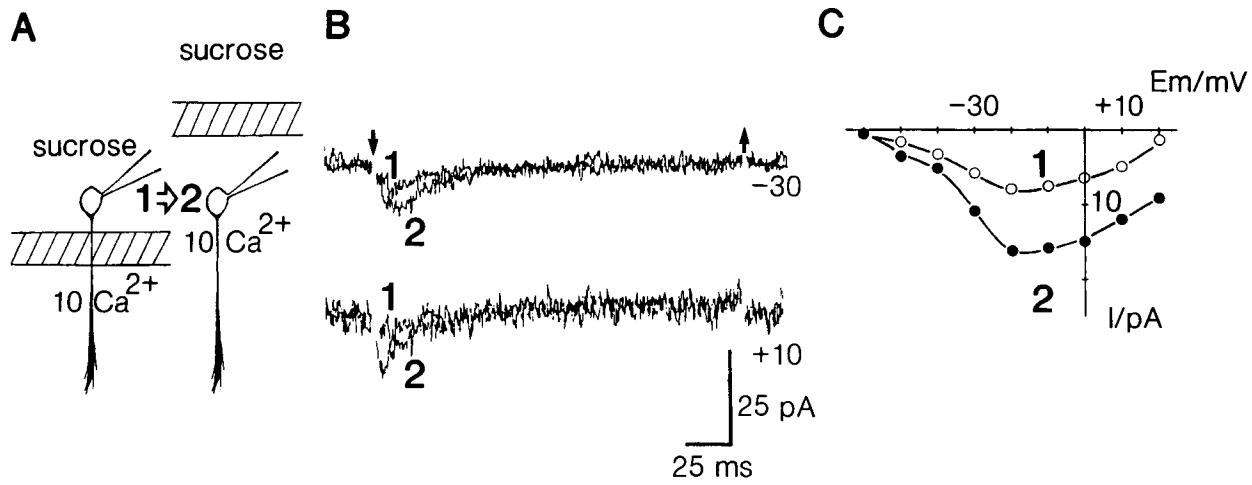

Figure 7. Distribution of LVA Ca^{2+} conductances in functionally undifferentiated precursor cells. *A*, First, growth cone conductances were isolated by superfusing soma and proximal neurite with sucrose (*1*). Then, the whole cell was exposed to 10 mM Ca^{2+} and 130 mM Na^+ (*2*). *B* and *C*, Current records (*B*) and *I/V* relationships (*C*). Holding potential, −80 mV. LVA Ca^{2+} conductances were present on the growth cone (*1*). After unblocking of somatic channels, LVA Ca^{2+} current amplitudes increased (*2*). HVA Ca^{2+} and Na^+ currents were not yet expressed by these cells. *Em*, membrane potential. *Arrows* indicate beginning and end of depolarizing pulse.

the absence of rapidly inactivating Na^+ currents. This ensured the functionally undifferentiated state of the cells. After a few hours in culture, these cells had already grown out neurites of about 50 μm in length and showed well-developed growth cones.

The distribution of LVA Ca^{2+} conductances was studied by first superfusing soma and proximal neurite with sucrose and recording growth cone currents. Then, the whole cell was exposed to Ca^{2+}-containing solution (Fig. 7*A*). Because of its short length, no attempt was made to demonstrate conductances located on the neuritic shaft.

Growth cone LVA Ca^{2+} currents were resolved in seven out of eight growth cones. Mean amplitude of growth cone LVA Ca^{2+} current was 7.8 ± 3.6 pA (−20 mV). Unblocking of somatic conductances resulted in an increased amplitude of LVA Ca^{2+} currents in all cells (n = 5) (Fig. 7*B,C*). Mean amplitude of whole-cell LVA Ca^{2+} current was 18.4 ± 5.1 pA (−20 mV). Time courses and voltage dependence were identical to isolated LVA Ca^{2+} currents in differentiated DRG neurons (Carbone and Lux, 1987).

Thus, even in functionally undifferentiated precursor cells, which did not yet express Na^+ currents, LVA Ca^{2+} channels were located on the growth cone membrane (Table 1).

Discussion

The distribution of Ca^{2+} and Na^+ channels in different parts (somatic, neuritic, and growth cone) of the cell membrane was investigated during neuronal differentiation. Conductances on the membrane area of interest were demonstrated in one of two ways: either (1) as isolated conductances after blocking all conductances on other membrane areas by superfusion with sucrose, or (2) as additional conductances after unblocking the channels on the neuritic shaft. For these reasons, the obtained results cannot be explained by run down of currents during successive measurements.

The electrotonic distance between somatic recording site and peripheral channels had to be short enough to permit voltage control of peripheral membrane areas. Currents did not exhibit abrupt activation, characteristic of inadequate space clamp. Using isolated somatic currents as controls, the *I/V* relationship did not show shifts along the voltage axis after unblocking of additional peripheral conductances (Fig. 3*C*). This suggests that a sufficient voltage control was achieved in the peripheral parts of the membrane.

The density of Ca^{2+} conductances in different parts of the cell

Table 1. Distribution of Ca^{2+} and Na^+ conductances at different stages of differentiation

Type of conductance	Soma	Neuritic shaft	Growth cone
Functionally undifferentiated precursor cells: 0–10 hr in culture			
LVA Ca^{2+}	++		++
During differentiation: 10–25 hr in culture			
LVA Ca^{2+}	++	+	++
HVA Ca^{2+}	++	++	++
Na^+	++	++	++

Functionally undifferentiated precursor cells did not express HVA Ca^{2+} and Na^+ conductances. Only cells that expressed a certain type of conductance on any part were included. +, Found in less than 80% of cells; ++, found in more than 80% of cells.

membrane of sprouting PC12 cells had been quantitatively determined (Streit and Lux, 1989). To calculate current densities, it is necessary to measure membrane surfaces. Because of the simple geometry of PC12 neurites and growth cones, this is quite possible from morphometric data (Streit and Lux, 1989). However, growth cones and neurites of DRG precursor cells show a much more complex geometry; in particular, growth cones exhibit numerous filopodia and the diameter of neurites changes along their length (Fig. 1), prohibiting a reliable surface estimation. Therefore, current densities were not calculated from the obtained current recordings.

In differentiated DRG neurons (after more than 25 hr in culture), voltage-dependent conductances (Na^+, Ca^{2+}) were located on the growth cone, neuritic, and somatic membranes. In regenerating DRG neurons (isolated at E10), Na^+, LVA, and HVA Ca^{2+} conductances in the somatic (Carbone and Lux, 1986, 1987) as well as in the growth cone membrane (Gottmann and Lux, 1990) have been demonstrated. Growth cone Na^+ channels in differentiated neurons have also been reported in PC12 cells (O'Lague et al., 1985). Growth cone Ca^{2+} channels have been demonstrated in numerous preparations of differentiated neurons by electrophysiological methods (Anglister et al., 1982; Streit and Lux, 1987, 1989, 1990; Haydon and Man-Son-Hing, 1988; Lipscombe et al., 1988) and by optical measurements of intracellular Ca^{2+} concentration (Bolsover and Spector, 1986; Cohan et al., 1987; Silver et al., 1990).

We have now investigated the distribution of Ca^{2+} and Na^+ channels in differentiating precursor cells. DRG precursor cells after 10–25 hr in culture, which have already grown out neurites of several 100 μm in length, are just starting to express HVA Ca^{2+} and Na^+ currents (Gottmann et al., 1988). We showed that HVA Ca^{2+} and Na^+ conductances were present on the somatic, neuritic, and growth cone membranes. Cells exhibiting HVA Ca^{2+} or Na^+ conductances only on the somatic membrane or only on the peripheral membrane were not found. In agreement with our results, PC12 cells showed HVA Ca^{2+} conductances on the somatic, neuritic, and growth cone membranes. However, NGF-primed, regenerating cells, which grow out neurites very rapidly, showed no HVA Ca^{2+} conductances on most parts of the neuritic shaft (Streit and Lux, 1989). This suggests that channel distribution in differentiating cells, which express channels after neurite outgrowth has occurred, is different from that of rapidly growing, regenerating cells.

Differentiating precursor cells also express LVA Ca^{2+} conductances (Gottmann et al., 1988). In cells cultured for 10–25 hr, LVA Ca^{2+} conductances were present on the somatic as well as on the growth cone membrane. This is in agreement with an investigation in regenerating DRG neurons (E10) demonstrating LVA Ca^{2+} conductances on the growth cone (Gottmann and Lux, 1990), but in contrast to earlier suggestions in other cell types that LVA Ca^{2+} channels are restricted to the somatic membrane (Llinas and Yarom, 1981; Yaari et al., 1987; Haydon and Man-Son-Hing, 1988). Overall, our results indicate that precursor cells differentiating in culture express Ca^{2+} and Na^+ channels on all parts of the surface membrane. It may be speculated that spatial restriction of channel expression occurs by interaction with the *in vivo* environment.

During neurite outgrowth of PC12 cells, the highest Ca^{2+} channel density of the entire cell is maintained in the growth cone (Streit and Lux, 1989), and we have now demonstrated the expression of Ca^{2+} and Na^+ channels on growth cones during differentiation. Growth cone action potentials (Gottmann and Lux, 1990) activate growth cone Ca^{2+} channels and increase intracellular Ca^{2+} concentration leading to inhibition of neurite growth (Kater et al., 1988; Fields et al., 1990; Holliday and Spitzer, 1990). By contrast, increases in intracellular Ca^{2+} seem to increase growth cone surface (Anglister et al., 1982; Goldberg, 1988; Silver et al., 1990).

Functionally undifferentiated DRG precursor cells (during the first 10 hr in culture) exhibit only LVA Ca^{2+} currents (Gottmann et al., 1988). We have now shown that LVA Ca^{2+} conductances are expressed on the growth cone membrane prior to the appearance of Na^+ conductances. LVA Ca^{2+} channels are activated by small depolarizations and are expressed prior to the development of electrical excitability (Gottmann et al., 1988) and prior to the expression of HVA Ca^{2+} currents (Yaari et al., 1987; Gottmann et al., 1988; McCobb et al., 1989). At this stage of differentiation, neurites were growing well, suggesting that HVA Ca^{2+} and Na^+ channels are not necessary for outgrowth. The early expression of LVA Ca^{2+} channels on growth cones may indicate a role in Ca^{2+}-dependent regulation of growth cone morphology and growth direction.

References

Anglister L, Farber IC, Shahar A, Grinvald A (1982) Localization of voltage-sensitive calcium channels along developing neurites: their possible role in regulating neurite elongation. Dev Biol 94:351–365.

Bolsover SR, Spector I (1986) Measurements of calcium transients in the soma, neurite, and growth cone of single cultured neurons. J Neurosci 6:1934–1940.

Carbone E, Lux HD (1986) Sodium channels in cultured chick dorsal root ganglion neurons. Eur Biophys J 13:259–271.

Carbone E, Lux HD (1987) Kinetics and selectivity of a low-voltage-activated calcium current in chick and rat sensory neurones. J Physiol (Lond) 386:547–570.

Carbone E, Morad M, Lux HD (1987) External Ni^{2+} selectively blocks the low-threshold calcium current of chick sensory neurons. Pfluegers Arch 408:R60.

Cohan CS, Connor JA, Kater SB (1987) Electrically and chemically mediated increases in intracellular calcium in neuronal growth cones. J Neurosci 7:3588–3599.

Fields RD, Neale EA, Nelson PG (1990) Effects of patterned electrical activity on neurite outgrowth from mouse sensory neurons. J Neurosci 10:2950–2964.

Goldberg DJ (1988) Local role of Ca^{2+} in formation of veils in growth cones. J Neurosci 8:2596–2605.

Gottmann K, Lux HD (1990) Low- and high-voltage-activated Ca^{2+} conductances in electrically excitable growth cones of chick dorsal root ganglion neurons. Neurosci Lett 110:34–39.

Gottmann K, Dietzel ID, Lux HD, Huck S, Rohrer H (1988) Development of inward currents in chick sensory and autonomic neuronal precursor cells in culture. J Neurosci 8:3722–3732.

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pfluegers Arch 391:85–100.

Haydon PG, Man-Son-Hing H (1988) Low- and high-voltage-activated calcium currents: their relationship to the site of neurotransmitter release in an identified neuron of *Helisoma*. Neuron 1:919–927.

Holliday J, Spitzer NC (1990) Spontaneous calcium influx and its roles in differentiation of spinal neurons in culture. Dev Biol 141:13–23.

Hume RI, Role LW, Fischbach GD (1983) Acetylcholine release from growth cones detected with patches of acetylcholine receptor-rich membranes. Nature 305:632–634.

Kater SB, Mattson MP, Cohan C, Connor J (1988) Calcium regulation of the neuronal growth cone. Trends Neurosci 11:315–321.

Lipscombe D, Madison DV, Poenie M, Reuter H, Tsien RY, Tsien RW (1988) Spatial distribution of calcium channels and cytosolic calcium transients in growth cones and cell bodies of sympathetic neurons. Proc Natl Acad Sci USA 85:2398–2402.

Llinas R, Yarom Y (1981) Properties and distribution of ionic conductances generating electroresponsiveness of mammalian inferior olivary neurones *in vitro*. J Physiol (Lond) 315:569–584.

McCobb DP, Best PM, Beam KG (1989) Development alters the expression of calcium currents in chick limb motoneurons. Neuron 2:1633–1643.

O'Lague PH, Huttner SL, Vandenberg CA, Morrison-Graham K, Horn R (1985) Morphological properties and membrane channels of the growth cones induced in PC12 cells by nerve growth factor. J Neurosci Res 13:301–321.

Rohrer H, Henke-Fahle S, El-Sharkawy T, Lux HD, Thoenen H (1985) Progenitor cells from embryonic chick dorsal root ganglia differentiate *in vitro* to neurons: biochemical and electrophysiological evidence. EMBO J 4:1709–1714.

Silver RA, Lamb AG, Bolsover SR (1989) Elevated cytosolic calcium in the growth cone inhibits neurite elongation in neuroblastoma cells: correlation of behavioral states with cytosolic calcium concentration. J Neurosci 9:4007–4020.

Silver RA, Lamb AG, Bolsover SR (1990) Calcium hotspots caused by L-channel clustering promote morphological changes in neuronal growth cones. Nature 343:751–754.

Streit J, Lux HD (1987) Voltage dependent calcium currents in PC12 growth cones and cells during NGF-induced cell growth. Pfluegers Arch 408:634–641.

Streit J, Lux HD (1989) Distribution of calcium currents in sprouting PC12 cells. J Neurosci 9:4190–4199.

Streit J, Lux HD (1990) Calcium current inactivation during nerve-growth-factor-induced differentiation of PC12 cells. Pfluegers Arch 416:368–374.

Tsien RW, Lipscombe D, Madison DV, Bley KR, Fox AP (1988) Multiple types of neuronal calcium channels and their selective modulation. Trends Neurosci 11:431–438.

Yaari Y, Hamon B, Lux HD (1987) Development of two types of calcium channels in cultured mammalian hippocampal neurons. Science 235:680–682.

Young SH, Poo M (1983) Spontaneous release of transmitter from growth cones of embryonic neurones. Nature 305:634–637.