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Extrinsic Factors Influence the Expression of Voltage-gated K Currents on Neonatal Rat Sympathetic Neurons

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Voltage-gated potassium (K) currents are important in controlling a neuron's excitability. We have shown previously (McFarlane and Cooper, 1992) that neonatal superior cervical ganglia (SCG) neurons express three voltage-gated K currents: a noninactivating delayed-rectifier type current (I_K), a rapidly inactivating A-current (I_{Af}), and a slowly inactivating A-current (I_{As}). When grown in culture for 4 weeks without other cell types, SCG neurons lose their expression of I_{Af} and I_{As}, suggesting that an extrinsic factor(s) is involved in controlling the expression of these currents. *In vivo*, SCG neurons are surrounded by non-neuronal cells. Therefore, in this study we investigated whether the ganglionic non-neuronal cells provide a factor required for A-current expression. We show that postnatal day 1 (P1) SCG neurons continue to express I_{Af} and I_{As} when cocultured with their ganglionic non-neuronal cells. Medium conditioned by ganglionic non-neuronal cells mimics the non-neuronal cell influence on I_{Af} and I_{As} expression, suggesting that the effects of non-neuronal cells are mediated by way of a secreted factor. Ciliary neurotrophic factor, a factor present in peripheral non-neuronal cells, has similar effects to those of ganglionic cell–conditioned medium. Moreover, we find that the dependence of I_{Af} on a non-neuronal cell factor is developmentally regulated; P14 neurons grown in culture without other cell types continue to express I_{Af}. However, I_{As} on P14 neurons maintains its dependence on a factor from non-neuronal cells. Finally, in addition to extrinsic control of voltage-gated K currents, we suggest that SCG neurons use intrinsic mechanisms to coordinate their expression of I_{Af}, I_{As}, and I_K such that changes in one K current are compensated for by reciprocal changes in one or more of the other K currents.

[Key words: K currents, extrinsic factors, sympathetic neurons, rat, non-neuronal cells, ciliary neurotrophic factor]

Many studies have examined the properties of K currents expressed on neurons (see reviews by Llinas, 1988; Rudy, 1988). From these investigations, it is clear that many different K currents exist and that the particular set a neuron expresses determines much of its electrophysiological behavior, particularly the frequency and pattern of action potential firing (Thompson and Aldrich, 1980; Adams and Galvan, 1986; Llinas, 1988). In view of the functional importance of K currents, it is surprising that more is not known about factors that govern their expression.

The K currents a neuron expresses could be controlled, in part, by a program intrinsic to the neurons. However, it is clear that epigenetic influences also have a role (O'Lague et al., 1978; Chalazonitis et al., 1987; Joels and de Kloet, 1989; Pragnell et al., 1990; Dourado and Dryer, 1992); one example of such epigenetic influences comes from our studies of neonatal rat sympathetic neurons (McFarlane and Cooper, 1992). These neurons express three voltage-gated K currents: a rapidly inactivating A-current (I_{Af}), a slowly inactivating A-current (I_{As}), and a noninactivating current (I_K). During the first 2 postnatal weeks, the expression of I_{Af} increases threefold and the expression of I_{As} decreases twofold. Yet, when neurons from 1-d-old (P1) animals develop in culture without other cell types for 2–3 weeks, their expression of I_{Af} and I_{As} falls to low levels and I_K increases fivefold. These results suggest that some factor(s), present *in vivo* but absent from the cultures, is necessary for the expression of I_{Af} and I_{As}. Others have also reported decreases in I_A in neurons in culture (Nerbonne and Gurney, 1989; Dourado and Dryer, 1992; Wu and Barish, 1992). Presumably, A-current expression could be controlled by either target tissues (Black and Mytilineou, 1976; Bunge et al., 1978; Patterson, 1978; Furshpan et al., 1982; Landis, 1990), or preganglionic nerves (Black et al., 1971; Black, 1982). However, we have shown that neither preganglionic denervation nor postganglionic axotomy affects the changing expression of voltage-gated K currents on developing sympathetic neurons (McFarlane and Cooper, 1992).

One possible influence could originate from ganglionic non-neuronal cells that are present *in vivo*, but absent from the cultures in our studies. Several reports indicate that ganglionic non-neuronal cells, or glia, can influence the differentiation of neurons (Bunge et al., 1978; Patterson, 1978; Black, 1982). In particular, O'Lague et al. (1978) demonstrated that neonatal rat sympathetic neurons increase their expression of a Ca-activated K conductance when cocultured with non-neuronal cells. Conceivably, factors from ganglionic non-neuronal cells could similarly affect the expression of voltage-gated K currents on these neurons. Therefore, the objective of this study was to investigate influences of ganglionic non-neuronal cells on the expression of voltage-gated K currents by neonatal sympathetic neurons.

Materials and Methods

All experiments were performed on dissociated sympathetic neurons derived from superior cervical ganglia (SCG) of 1-d-old (P1), or 14-d-old (P14) rat pups. The methods used to dissociate the neurons were

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similar to those described previously (McFarlane and Cooper, 1992). Briefly, SCG were dissected from rat pups (CD strain, Charles River Canada); the ganglia were placed in enzyme-containing media (Dispase, grade 2, Boehringer Mannheim; 2.4 mg/ml in Hank's Balanced Salt Solution) at 37°C for 3 hr, and dissociated by gentle trituration with a fire-polished pipette every 15 min. The dissociated neurons were transferred to growth media consisting of L-15 (Flow Lab.) supplemented with vitamins, cofactors, rat serum (5%), and 7S nerve growth factor (1 μg/ml) (Bocchini and Angeletti, 1969). In one series of experiments, recombinant rat ciliary neurotrophic factor (CNTF; 10 ng/ml; kindly provided by Dr. P. Richardson, McGill University) was added to the growth media. The neurons were plated on Aclar (Allied Chemicals) coverslips coated with rat tail collagen and laminin (50 μg/ml; courtesy of Dr. S. Carbonetto, McGill University). The cultures were maintained for 2–4 weeks at 37°C in 95% air, 5% CO_2 and fed every 3–4 d with the growth media described above. In some experiments, recordings were done on acutely dissociated P1 or P14 neurons.

Neuronal cultures. For neuronal cultures essentially free of non-neuronal cells, most of the non-neuronal cells were removed from the ganglionic cell suspension after dissociation by centrifugation through a density gradient (33% Percoll, Pharmacia). In addition, cytosine arabinoside (ARA-C; Sigma) (5–10 μM) was added to the growth media for the initial 2–3 d, which essentially eliminates all the remaining non-neuronal cells.

Explant cultures. SCG were removed from P1 animals and cut in half, and the pieces were maintained in culture for 2 weeks, as for dissociated neuron cultures. Thereafter, the cultures were removed and the neurons dissociated enzymatically, as described for freshly isolated ganglia, in order to obtain high-resistance seals with the patch electrodes. Whole-cell recordings were made from these neurons within 2–24 hr of dissociation.

Cocultures of SCG neurons with ganglionic non-neuronal cells. SCG neurons from P1 or P14 animals were plated on monolayers made from ganglionic non-neuronal cells, and cocultured for 14–28 d. For P1 neurons, the non-neuronal cells were from P1 ganglia and for P14 neurons the non-neuronal cells were from P14 ganglia. Non-neuronal cells were obtained from the supernatant after centrifuging the ganglionic cell suspension through a Percoll density gradient. The non-neuronal cells were plated on laminin/collagen-coated coverslips, fed with growth media consisting of L15-CO_2 medium, penicillin–streptomycin, glutamine, glucose, vitamins, cofactors, and fetal calf serum (10%), until a confluent monolayer had formed (usually 7–10 d); then cells were irradiated (^{60}Co, 5000 cGy) to prevent further cell division. In some experiments, neurons and non-neuronal cells were cocultured together from the time of plating by omitting the Percoll gradient and by not treating the cultures with ARA-C. In these cultures, the non-neuronal cells formed a monolayer by 1 week. We observed no difference in the K currents expressed by the neurons when cocultured with non-neuronal cells by either method.

Conditioned media. Alternatively, P1 neurons were grown in the absence of non-neuronal cells, but in the presence of media conditioned by ganglionic non-neuronal cells. Conditioned medium (CM) was obtained according to the method of Patterson and Chun (1977). Briefly, ganglionic non-neuronal cells were grown to confluency, and the media were harvested every 3–4 d, passed through a 0.2 μm filter (Nucleopore), and stored at −20°C. SCG neurons from P1 animals were grown in culture in the presence of 60% CM, 40% growth media; NGF, rat serum (5%), penicillin–streptomycin, glucose, glutamine, vitamins, and cofactors were increased by 60% as if they were not present in the CM. Control cultures (without CM) also had the ingredients of their growth media increased by 60%; this had no effect on the K currents normally expressed by cultured neurons. As previously reported (Patterson and Chun, 1977), CM had no effect on the survival of SCG neurons in culture.

Data recording and analysis. SCG neurons were voltage clamped using whole-cell recording techniques (Hamill et al., 1981). All experiments were done at room temperature (21–24°C) with a List EPC-7 amplifier. Pipette resistances were 2–5 MΩ and were filled with intracellular media (described below). The current signal was balanced to zero with the pipette immersed in the bathing solution. The seal resistances were usually 5–20 GΩ, and the series resistances (6–10 MΩ) were usually partly compensated (20–30%). A 386-based PC computer [AT class with an EISA bus and running at 33 MHz with a 64K cache and an A/D card (Omega)] was used to deliver the voltage-clamp steps and acquire the membrane currents; the software for stimulation, data acquisition, and analysis was written by Mr. A. Sherman (Alembic Inc., Montreal). Membrane currents and voltages were filtered at 3 kHz with an eight-pole Bessel filter (Frequency Devices, Inc.), sampled, displayed, and stored on line. The durations of the voltage steps were either 125 msec, or 6 and 10 sec: for 125 msec steps the data were sampled at 5–10 kHz, whereas for the 6 and 10 sec steps, the data were filtered at 100 Hz and sampled at 200 Hz.

To study voltage-gated K currents, inward and Ca-dependent currents were blocked pharmacologically: Na currents were blocked with TTX (1 μM), and the external Na concentration was reduced to 2 mM and replaced by 140 mM choline chloride; Ca currents were blocked by $CoCl_2$ (2.0 mM) and by lowering the external Ca concentration to 0.5 mM. Furthermore, any neuron in which there was a suggestion of inadequate space clamp, such as notches or oscillations, was excluded from this study.

The voltage-gated outward current on SCG neurons is made up of three different K currents (McFarlane and Cooper, 1992): a noninactivating current (I_K), a fast transient A-current (I_{Af}), and a slow transient A-current (I_{As}). The method used to separate these three K currents has been described previously (McFarlane and Cooper, 1992; see also McFarlane and Cooper, 1991). Briefly, the three currents were isolated as follows. For I_K, a depolarizing voltage step to +30 mV was delivered from a V_h of −10 mV; at this potential, I_K can be activated, whereas both I_{Af} and I_{As} are inactivated. I_K was corrected for leakage and capacity currents by digitally adding the current evoked by an equivalent hyperpolarizing step. For I_{As}, I_K evoked by depolarizing step to +30 mV, from a V_h of −10 mV, was subtracted from the outward current (I_{As} and I_K) evoked by a depolarizing step to +30 mV from a V_h of −40 mV (or −90 mV), and the current evoked by a hyperpolarizing step from −10 to −40 mV (or −90 mV) was added to the record to remove the leakage current. For I_{Af}, currents evoked by a depolarizing voltage step to +30 mV, from a V_h of −40 mV (I_{As} and I_K), were subtracted from the corresponding current evoked from a V_h of −90 mV (I_{Af}, I_{As}, and I_K). The current evoked by a hyperpolarizing step from −40 to −90 mV was added to the trace. The voltage dependence of inactivation for I_{As} on neurons grown with non-neuronal cells, ciliary neurotrophic factor (CNTF), or conditioned media was similar to P14 neurons, 15 mV more positive than for P1 neurons. However, this shift did not affect our measurements of I_{As}, which was always taken as the maximal I_{As} current. The voltage dependence of activation and inactivation for I_{Af} and I_K was the same in the different culture conditions.

To quantify the expression of each current, I_{Af}, I_{As}, and I_K amplitudes for each neuron were measured and normalized to membrane capacitance. The amplitudes were determined from the current evoked by a voltage step to +30 mV after each current was isolated from the other two as described above. For I_{Af}, measurements were made at the peak current, which occurred within the first 10 msec. For I_{As}, measurements were made at the plateau, 125 msec after the beginning of the step. Measurements of I_K were made at the end of a 10 sec voltage step to +30 mV, at which time I_{As} had completely decayed. The membrane capacitance (pF) was obtained by integrating the capacitative current evoked by a 10 mV hyperpolarizing voltage step. Standard *t* tests were used to compare differences between mean current densities.

Solutions. The ionic composition of the extracellular solution was 140 mM choline Cl, 2 mM NaCl, 5.4 mM KCl, 0.5 mM $CaCl_2$, 0.18 mM $MgCl_2$, 10 mM HEPES (pH 7.4, adjusted with NaOH; BDH), 5.6 mM glucose, 1 μM TTX (Sigma), and 2 mM $CoCl_2$. The pH was 7.3–7.4. The intracellular pipette solution contained 5 mM NaCl, 140 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES (pH 7.4, adjusted with KOH), 10 mM EGTA, and 0.2 mM $CaCl_2$ (final free Ca concentration < 10^{-8} M). The pH was 7.3–7.4.

Results

Figure 1*A* shows the total outward current from a rat SCG neuron isolated from a P1 animal, after inward and Ca-activated currents were blocked pharmacologically. The outward current was evoked by depolarizing voltage steps, 125 msec (left) and 6 sec (right) in duration. As shown previously, the voltage-gated outward current consists of three separate K currents: I_{Af}, I_{As}, and I_K (McFarlane and Cooper, 1992). I_{As} is the predominant current on P1 neurons. I_{As} shows little inactivation over 125 msec, but is seen to inactivate slowly over the course of 6 sec. The rapidly inactivating current, I_{Af}, at +30 mV, is on average

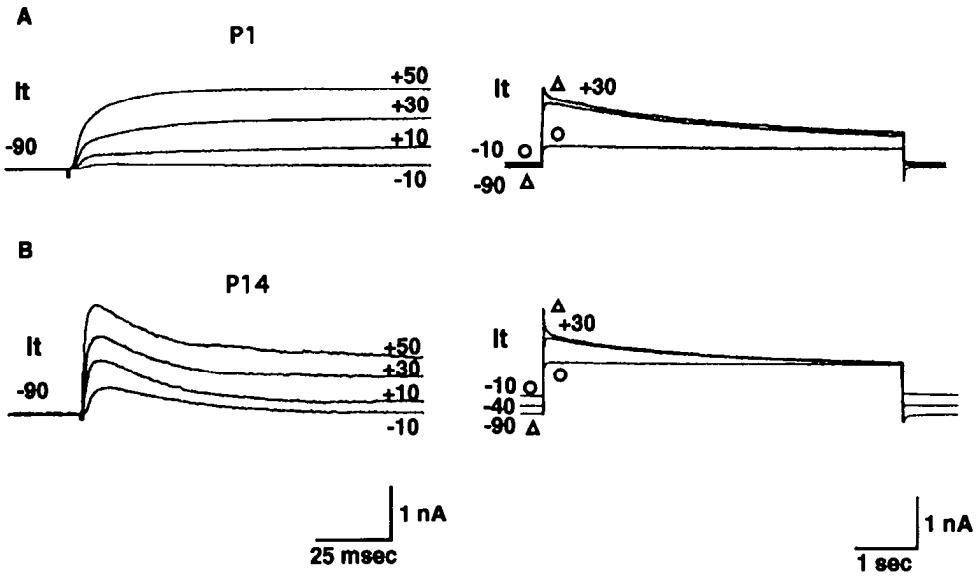

Figure 1. Voltage-gated K currents on SCG neurons: total outward currents expressed by acutely dissociated P1 (*A*) and P14 (*B*) SCG neurons. Outward currents were evoked by 125 msec depolarizing voltage steps in 20 mV increments to +50 mV from a V_h of −90 mV (*left*), and by 6 sec depolarizing steps to +30 mV (*right*) from a V_h of −90 mV (*triangles*), −40 mV, and −10 mV (*circles*). *A*, The P1 neuron expressed all three voltage-gated K currents, with I_{As} as the predominant current. *B*, The P14 neuron expressed a large I_{Af}, with smaller I_{As} and I_K currents. *Current traces on the left* were corrected for leakage and capacity currents, and were filtered at 1.5 kHz and sampled at 5 kHz. *Traces on the right* were not corrected for leakage current, and were filtered at 100 Hz and sampled at 200 Hz.

only one-third the size of I_{As}. I_K, which can be seen in isolation when a voltage step is given from a V_h of −10 mV, is the smallest current, and its amplitude at +30 mV is one-fifth that of I_{As}. Over the first 2 weeks of postnatal development, I_{Af} current densities increase threefold, I_{As} densities decrease by 40%, whereas I_K densities remain unchanged (McFarlane and Cooper, 1992). An example of the total voltage-gated outward current from a rat SCG neuron isolated from a 2-week-old (P14) animal is shown in Figure 1*B*.

K currents on SCG neurons cocultured with non-neuronal cells

Contrary to development *in vivo*, I_{Af} and I_{As} on P1 SCG neurons decrease significantly when the neurons develop in culture in the absence of other cell types, suggesting that some factor(s) necessary for the expression of these currents is missing in these cultures (McFarlane and Cooper, 1992). One possibility is that non-neuronal cells from the ganglion provide a factor(s) that affects A-current expression. To test this, we measured K current expression on P1 SCG neurons grown in culture with or without ganglionic non-neuronal cells. Figure 2 shows examples of the outward currents evoked by depolarizing voltage steps for two representative P1 neurons, one cultured without non-neuronal cells for 28 d (Fig. 2*A*), and the other from a sister culture where the neurons were cocultured with non-neuronal cells (Fig. 2*B*). Unlike neurons that develop without other cell types, the neurons cocultured with ganglionic non-neuronal cells continue to express both I_{Af} and I_{As}.

Figure 2, *C* and *D*, shows the mean current densities (pA/pF) for I_{Af}, I_{As}, and I_K for SCG neurons cocultured with or without non-neuronal cells for 2 and 4 weeks. For neurons cultured without non-neuronal cells (Fig. 2*C*), I_{Af} and I_{As} densities dropped to low levels (<15 pA/pF) by 4 weeks, whereas I_K increased fivefold (McFarlane and Cooper, 1992). In contrast, when neurons were cocultured with non-neuronal cells (Fig. 2*D*), I_{Af} and I_K densities remained at their P1 levels and did not change significantly with time in culture ($p > 0.1$, t test). Similarly, after 4 weeks, I_{As} current densities, which decreased 30–40% by 2 weeks ($p < 0.01$), were fourfold greater than on neurons grown without non-neuronal cells. These results indicate that non-neuronal cells can influence the expression of the three voltage-gated K currents.

In the above experiments, the neurons and non-neuronal cells were dissociated first and then cocultured. In some experiments, we cultured P1 neurons as explants to minimize the disruption of the *in vivo* relationship between the neurons and the non-neuronal cells. After 2 weeks, I_{Af} and I_K densities on the neurons in the explants were not significantly different ($p > 0.1$, t test) from those of P1 neurons cocultured with ganglionic non-neuronal cells in dissociate cultures, as shown by the open symbols in Figure 2*D*. I_{As} densities were 30% larger than for neurons from cocultures with non-neuronal cells ($p < 0.001$), and in fact, were comparable to I_{As} densities on P1 neurons ($p > 0.2$).

Non-neuronal cell-conditioned media affect the expression of K currents on SCG neurons in culture

To test whether non-neuronal cells exert their effects by a factor secreted into the media, we grew P1 SCG neurons without non-neuronal cells, but in the presence of medium that was first conditioned by ganglionic non-neuronal cells. Figure 3 shows the mean current densities (pA/pF) for I_{Af}, I_{As}, and I_K on P1 SCG neurons grown in culture for 28 d in different conditions; the absence of non-neuronal cells, cocultured with ganglionic non-neuronal cells, or cultured with media conditioned by non-neuronal cells (CM). There is no significant difference ($p > 0.1$) between the levels of I_{Af}, I_{As}, and I_K on P1 SCG neurons grown either in the presence of non-neuronal cells, or in the presence of media conditioned by these cells. These results suggest that non-neuronal cells provide a soluble factor that influences the expression of K currents on cultured P1 SCG neurons.

CNTF influences the expression of voltage-gated K currents

CNTF is found in high concentrations in non-neuronal cells of the rat sciatic nerve (Lin et al., 1989) and has been shown to influence the differentiation of sympathetic neurons (Saadat et al., 1989). This raised the possibility that CNTF may be present in the CM and influences the expression of I_{Af}, I_{As}, and I_K. Therefore, we tested the effects of CNTF on the expression of K currents by P1 SCG neurons developing in culture. As shown in Table 1, I_{Af}, I_{As}, and I_K densities on neurons cultured in the presence of 10 ng/ml of CNTF were not significantly different

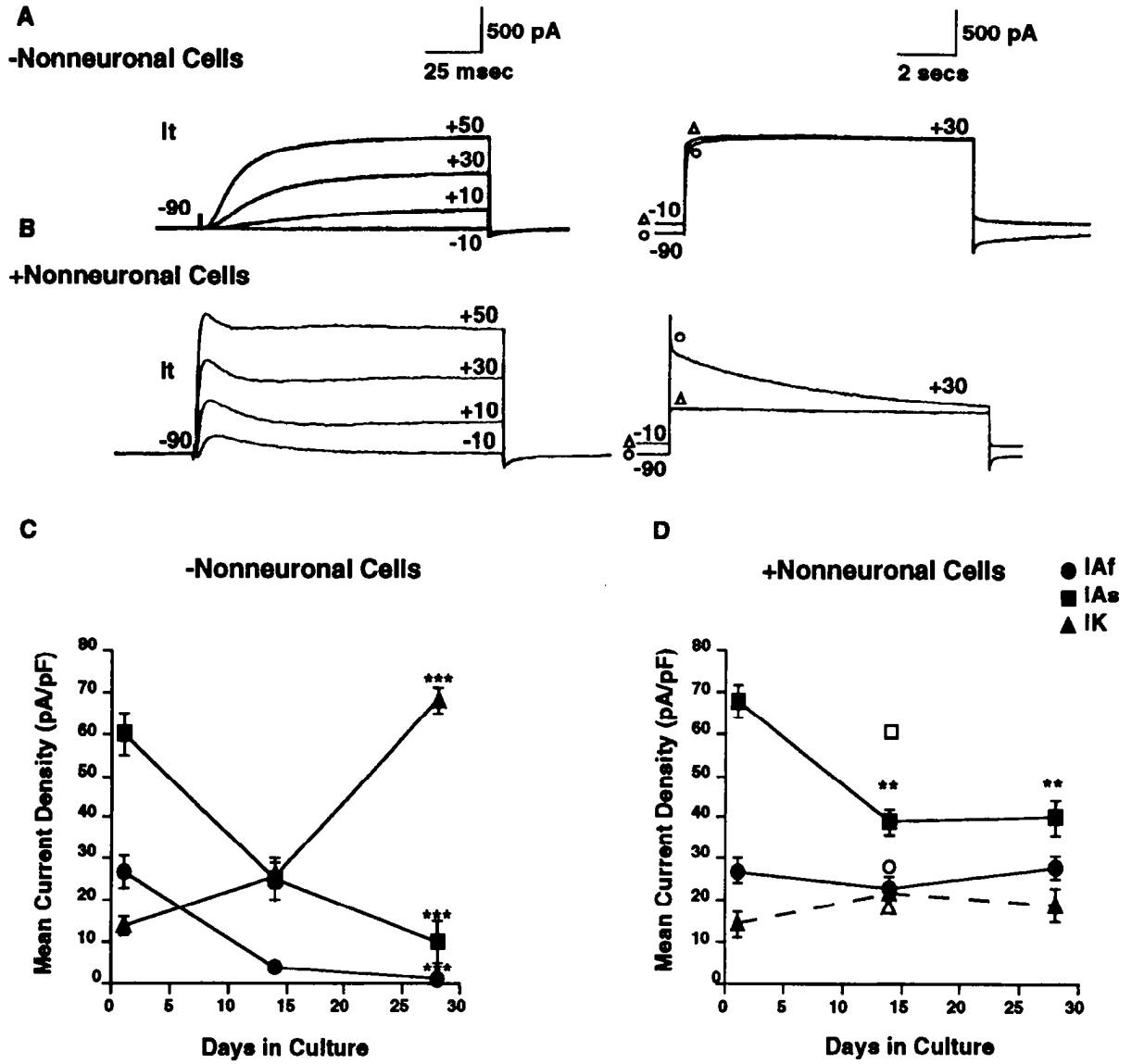

Figure 2. Non-neuronal cells influence K current expression in culture. *A* and *B*, Outward K currents expressed on P1 SCG neurons grown in culture for 28 d, in the absence or presence of other cell types from the ganglion. The neuron cultured in the absence of other cell types (*A*) expresses only I_K, while the neuron in coculture (*B*) expresses all three K currents. *Left*, Leak subtracted voltage-gated outward currents evoked by 125 msec depolarizing steps in 20 mV increments up to +50 mV, from a V_h of −90 mV. Currents were filtered at 1.5 kHz and sampled at 5 kHz. *Right*, Non-leak-corrected outward currents evoked by 10 sec depolarizing steps to +30 mV from a V_h of −90 mV (*circles*) and −10 mV (*triangles*). Traces were filtered at 100 Hz and sampled at 200 Hz. *C* and *D*, Mean current densities (pA/pF) for I_{Af}, I_{As}, and I_K expressed on P1 neurons after 1, 14, and 28 d in culture with or without ganglionic non-neuronal cells. *C*, Mean I_{Af} and I_{As} current densities fall (***, $p < 0.001$) and I_K increases (***, $p < 0.001$) significantly for P1 neurons grown without other cell types. P1, $n = 46$; C14, $n = 32$; C28, $n = 18$. *D*, When P1 neurons are cocultured with non-neuronal cells there is no loss of I_{Af} ($p > 0.2$) and no increase in I_K ($p > 0.1$). I_{As} drops by 30–40% (**, $p < 0.01$), but is fourfold greater ($p < 0.001$) than on neurons without non-neuronal cells. C14, $n = 58$; C28, $n = 54$. *Open symbols* represent the mean current densities for I_{Af}, I_{As}, and I_K measured for P1 neurons grown in explant cultures for 14 d ($n = 38$). Errors are SEM and in some cases are smaller than the symbols.

($p > 0.2$) from those on neurons in the presence of media conditioned by the ganglionic non-neuronal cells.

P14 neurons grown in the absence of other cell types

We previously showed that I_{Af} undergoes a significant increase in expression over the first 2 postnatal weeks (McFarlane and Cooper, 1992), so that the total voltage-gated K currents on P14 neurons resemble those found in the adult (Galvan and Sedlmeir, 1984; Belluzzi et al., 1985). As such, the disappearance of I_{Af} and I_{As} from P1 neurons in culture without other cell types could be because the adult expression of A-currents has not yet been established. Therefore, we were interested to know if P14 neurons would continue to express A-currents in culture under similar conditions. Figure 4*A* shows the K currents for a P14 neuron that has developed in culture for 28 d without other cell types, and demonstrates that P14 neurons continue to express I_{Af} when grown in culture. Figure 4*C* shows the mean I_{Af} current densities with time in culture, and demonstrates that while I_{Af} decreases by about 30% over the first 2 weeks in culture, thereafter its expression remains constant. This is in contrast to the loss of I_{Af} that is observed when P1 neurons are grown without non-neuronal cells, and suggests that over the first 2 postnatal

Table 1. Ciliary neurotrophic factor (CNTF) affects the expression of voltage-gated K currents on P1 SCG neurons developing in culture

	I_{Af} (pA/pF)	I_{As} (pA/pF)	I_K (pA/pF)
Conditioned media	23.2 ± 3	46.2 ± 6	22.4 ± 3
CNTF	22.9 ± 3	38.2 ± 3	16.5 ± 1

Mean current densities (pA/pF) for I_{Af}, I_{As}, and I_K on P1 neurons grown in culture for 14 d in the presence of non-neuronal cell-conditioned media (CM; $n = 35$), or in the presence of CNTF (10 ng/ml; $n = 58$). The levels of I_{Af}, I_{As}, and I_K are not significantly different on neurons grown in the presence of CNTF or in the presence of conditioned media from ganglionic non-neuronal cells. Errors are SEM.

weeks *in vivo* I_{Af} expression not only increases, but loses its dependence on a non-neuronal cell factor.

While cultured P14 neurons continue to express I_{Af}, the majority of P14 neurons express no detectable I_{As} after 4 weeks in culture (Fig. 4*A*). Figure 4*C* indicates that after 2 weeks in culture the mean I_{As} density on P14 neurons drops by 60%, and continues to decrease such that after 4 weeks many neurons express little or no I_{As} (< 10 pA/pF). This is similar to what is observed for I_{As} on P1 neurons grown in culture without other cell types, and suggests that unlike I_{Af}, I_{As} expression on P14 neurons is still dependent on a factor that is missing in these cultures. Also, similar to what is observed for P1 neurons cultured without their non-neuronal cells, the mean I_K density on P14 neurons increases fivefold over the 4 weeks in culture.

P14 neurons grown in the presence of ganglionic non-neuronal cells

When P1 SCG neurons are cocultured with non-neuronal cells from the ganglion, they continue to express I_{Af} and I_{As}. As such, we were interested to know if a factor from non-neuronal cells could similarly influence I_{Af} and I_{As} expression on P14 neurons. Therefore, we grew P14 neurons in culture with their non-neuronal cells for 2–4 weeks. Figure 4*B* shows the outward currents from a P14 neuron that has been cocultured for 28 d with ganglionic non-neuronal cells; this neuron expressed all three voltage-gated K currents, I_{Af}, I_{As}, and I_K. In fact, in two platings ($n = 50$), after 4 weeks 50% of the neurons expressed I_{As} at levels comparable to those found on P14 neurons *in vivo* (>30 pA/pF). These results suggest that the expression of I_{As} by P14 neurons depends on a factor provided by non-neuronal cells, as for P1 neurons. In a third plating ($n = 21$), only 9% of the neurons expressed I_{As} at >30 pA/pF; possibly, the density of non-neuronal cells in these cultures was not sufficient to maintain I_{As} on these neurons. In fact, the neurons in this plating expressed the three K currents at levels comparable to those on P14 neurons grown in culture without non-neuronal cells, and therefore, the data from this plating were not pooled with data from the two platings that did respond to non-neuronal cells.

Figure 4*D* shows the mean current densities (pA/pF) for I_{Af}, I_{As}, and I_K for the two platings where neurons responded to a

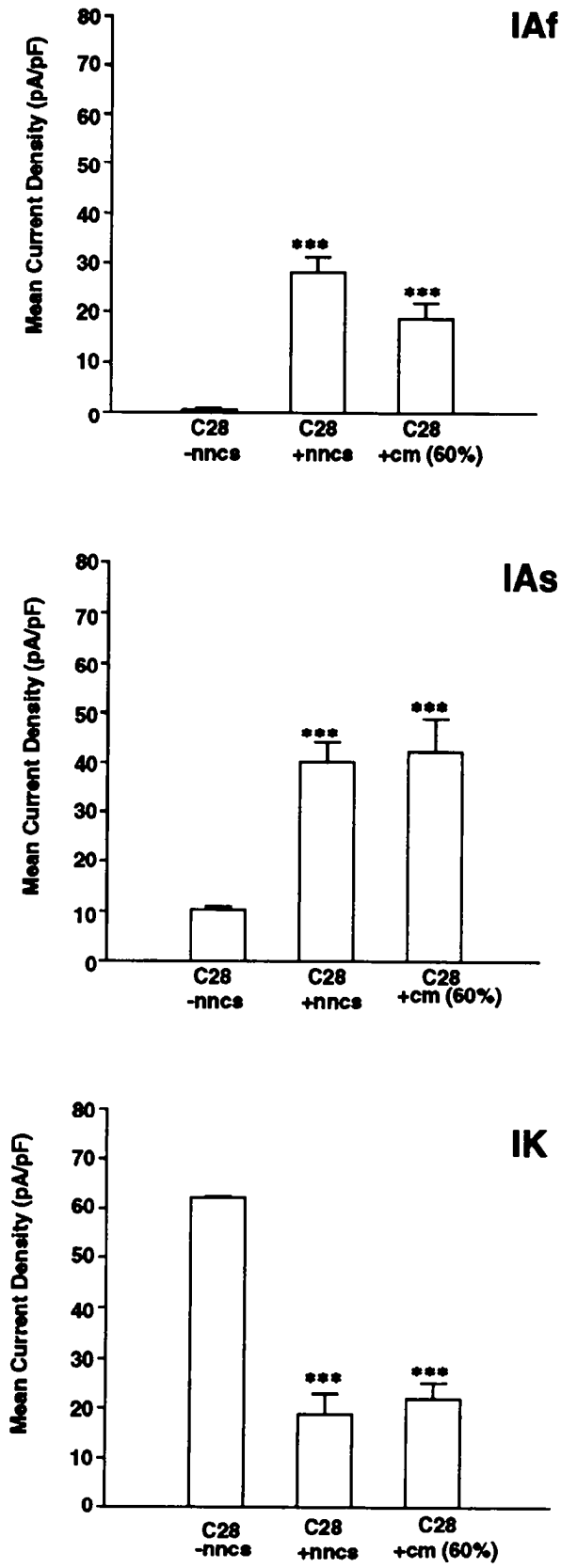

Figure 3. Conditioned medium from non-neuronal cells mimics the effects of non-neuronal cells: mean current densities (pA/pF) for I_{Af}, I_{As}, and I_K on P1 neurons grown in culture for 28 d in the presence ($n = 54$) and absence ($n = 35$) of non-neuronal cells from the ganglion, and on P1 neurons grown in the presence of conditioned media harvested from non-neuronal cell cultures ($n = 44$). There is no significant difference ($p > 0.1$, t test) between the levels of I_{Af}, I_{As}, and I_K expressed in P1 neurons grown either in the presence of non-neuronal cells, or in media conditioned by non-neuronal cells. In both cases, I_{Af} and I_{As} are significantly greater (***, $p < 0.001$) and I_K significantly smaller ($p < 0.001$) than the currents on neurons in culture without other cell types. Errors are SEM.

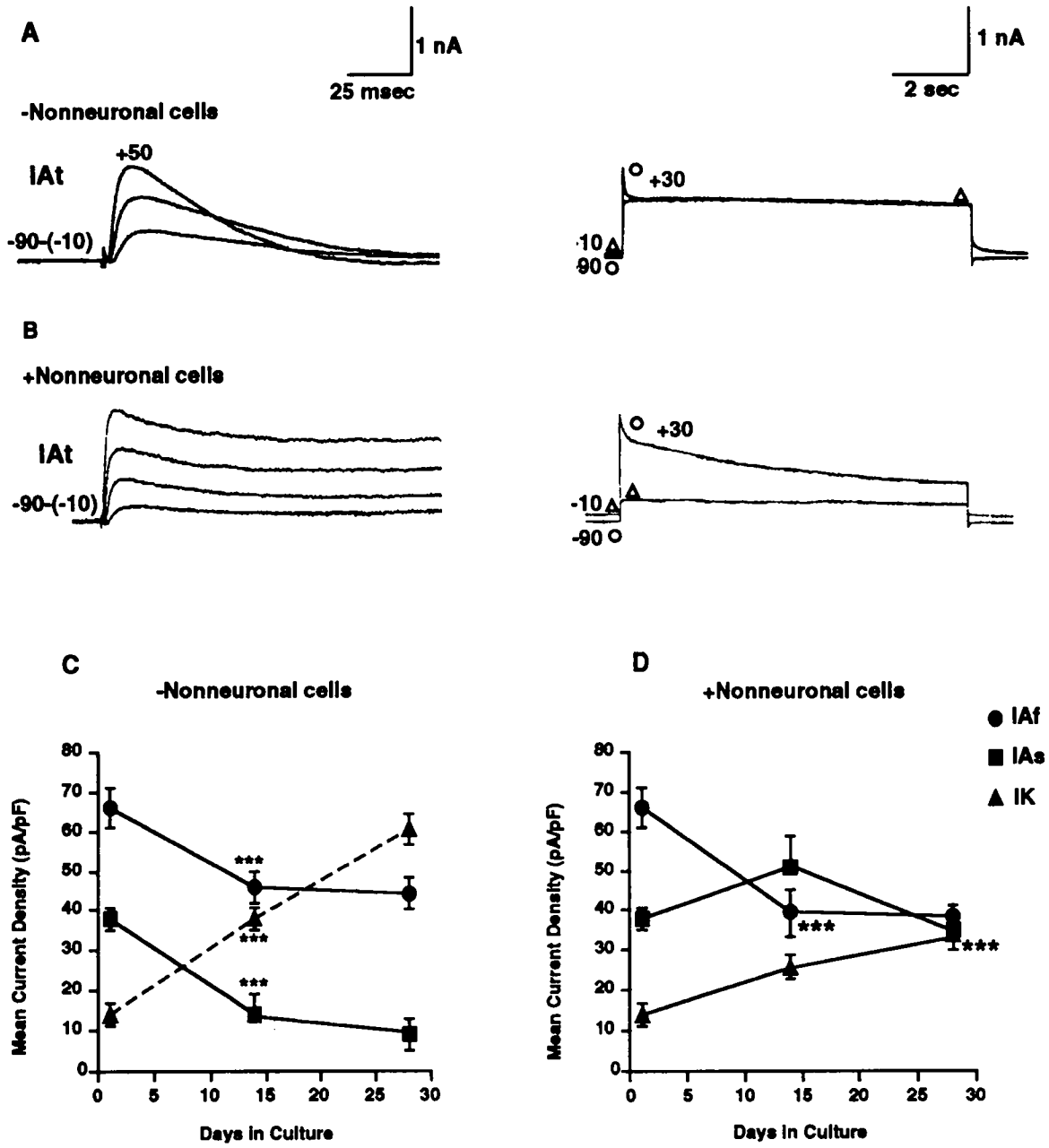

Figure 4. Expression of K currents on P14 SCG neurons in culture. *A* and *B* show outward currents from representative P14 neurons grown in culture for 28 d without other cell types (*A*), or with ganglionic non-neuronal cells (*B*). *Left*, Total A-current (I_{A_f}): currents were evoked by depolarizing voltage steps in 20 mV increments up to +50 mV from a V_h of −90 mV. I_K currents evoked by depolarizing steps to the same command potentials, from a V_h of −10 mV, have been subtracted to isolate the total A-current. Currents were filtered at 1.5 kHz and sampled at 5 kHz. *Right*, Non-leak-subtracted total outward current evoked by a 10 sec depolarizing step to +30 mV from V_h values of −90 mV (*circles*) and −10 mV (*triangles*). Currents were filtered at 100 Hz and sampled at 200 Hz. *C* and *D*, Mean current densities for I_{A_f}, I_{A_s}, and I_K on P14 neurons grown in culture for 2–4 weeks without other cell types (*C*) or with non-neuronal cells (*D*). When P14 neurons are grown in the absence of other cell types, I_{A_f} drops by a third (***, $p < 0.001$), I_K increases fivefold ($p < 0.001$), while I_{A_s} on most neurons drops to low levels. C1, $n = 62$; C14, $n = 31$; C28, $n = 35$. When P14 neurons are grown in the presence of non-neuronal cells from the ganglion, I_{A_f} levels drops by a third ($p < 0.001$) after 2 weeks in culture, and thereafter the levels remain constant; I_{A_s} levels are maintained; and I_K levels increase two- to threefold. C14, $n = 28$; C28, $n = 50$.

factor provided by non-neuronal cells. When neurons are grown with their non-neuronal cells, the mean I_{A_s} current density is >30 pA/pF, even after 4 weeks in culture, suggesting that I_{A_s} expression by P14 neurons in culture, as for P1 neurons, is sensitive to a factor provided by non-neuronal cells. In addition, I_K levels are twofold lower on P14 neurons cocultured with non-neuronal cells than on neurons in sister cultures grown for 28 d without non-neuronal cells ($p < 0.001$). In contrast, P14 neurons grown with or without their non-neuronal cells show a similar 30–40% drop in their mean I_{A_f} density ($p > 0.2$), supporting the idea that I_{A_f} expression is no longer dependent on a non-neuronal cell factor.

SCG neurons coordinate their expression of I_{A_f}, I_{A_s}, *and* I_K

Figure 5 shows representative examples of the voltage-gated outward K currents expressed by P1 and P14 SCG neurons

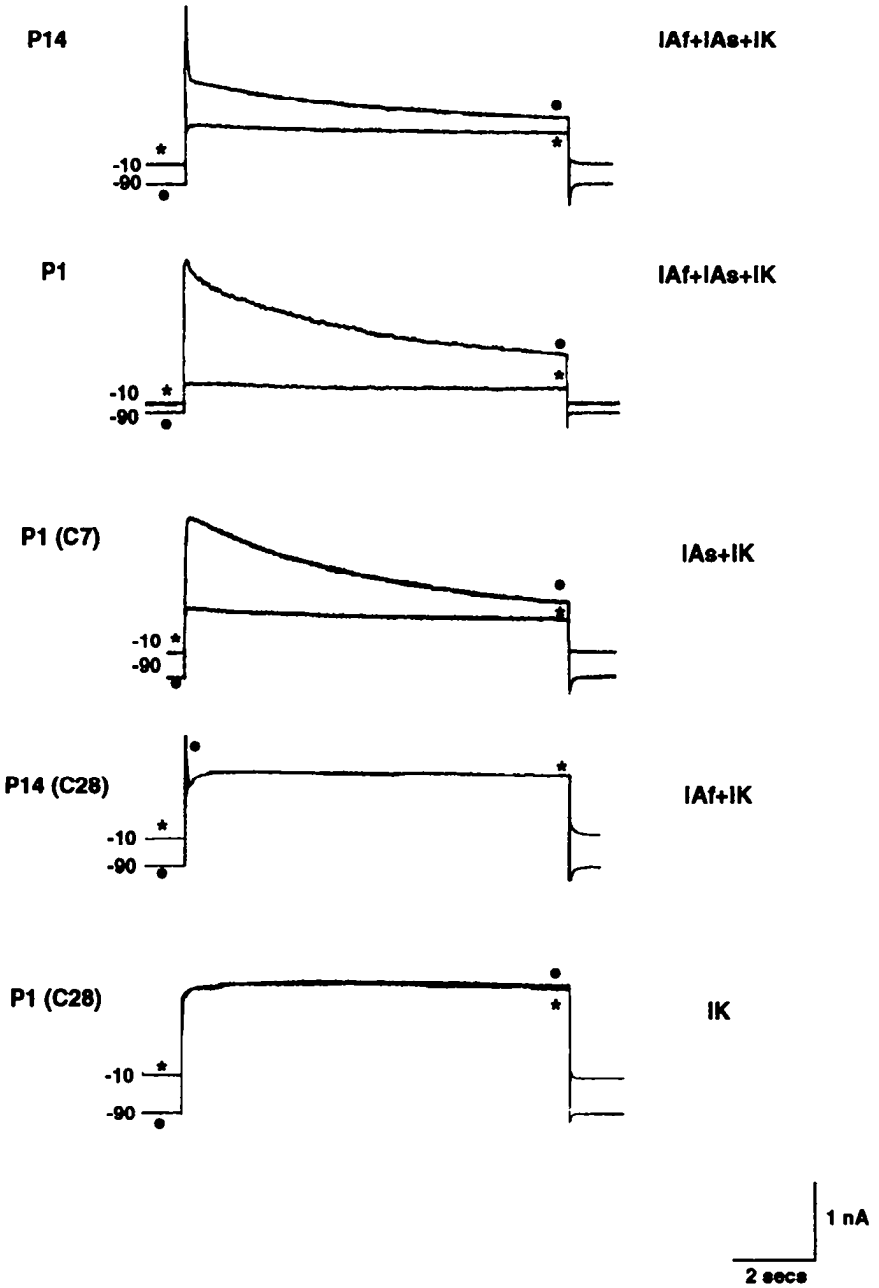

Figure 5. Representative examples of outward K currents for P1 and P14 neurons, and P1 and P14 neurons grown in culture without non-neuronal cells for different periods of time. Ten-second voltage steps to +30 mV were given from a V_h of −10 mV (*asterisks*) and −90 mV (*circles*). Currents were not corrected for leakage and were filtered at 100 Hz and sampled at 200 Hz.

maintained in culture without non-neuronal cells for different periods of time, and demonstrates that the relative proportion of I_{Af}, I_{As}, and I_K varies for SCG neurons depending on their developmental age and environment: some postnatal SCG neurons express all three K currents (P1 and P14) but at different relative levels, other neurons express only two of the voltage-gated K currents (P1 in culture for 7 d, and P14 in culture for 28 d), while a third group only expresses I_K (P1 in culture for 28 d). Since the relative proportions of I_{Af}, I_{As}, and I_K on SCG neurons will influence these neurons' electrical behavior, we are interested to know if the neurons control I_{Af}, I_{As}, and I_K levels in an interdependent fashion. Figure 6 summarizes our results on the expression of I_{Af}, I_{As}, and I_K on P1 and P14 neurons developing *in vivo* or in culture, and suggests that the neuron coordinates its expression of I_{Af}, I_{As}, and I_K such that changes in one K current are compensated for by reciprocal changes in one or more of the other K currents. For SCG neurons developing *in vivo*, we observed a reciprocal relationship in the expression of I_{Af} and I_{As}; initially, I_{As} is high and decreases with postnatal development, whereas I_{Af} is initially low and increases over the same period. In contrast, when P1 or P14 neurons develop in culture without other cell types, I_K levels increase at the same time as large decreases in one or two of the A-currents. However, when P1 neurons are cocultured with non-neuronal cells the neurons continue to express A-currents and I_K remains low. Similarly, when the total A-current ($I_{As}+I_{Af}$) does not change significantly, as is the case for *in vivo* development, I_K remains constant.

If the level of each voltage-gated K current is independently controlled, one would expect that the density of a given current, for example I_K, would not depend on the densities of the other two currents; however, we observed an inverse relationship be-

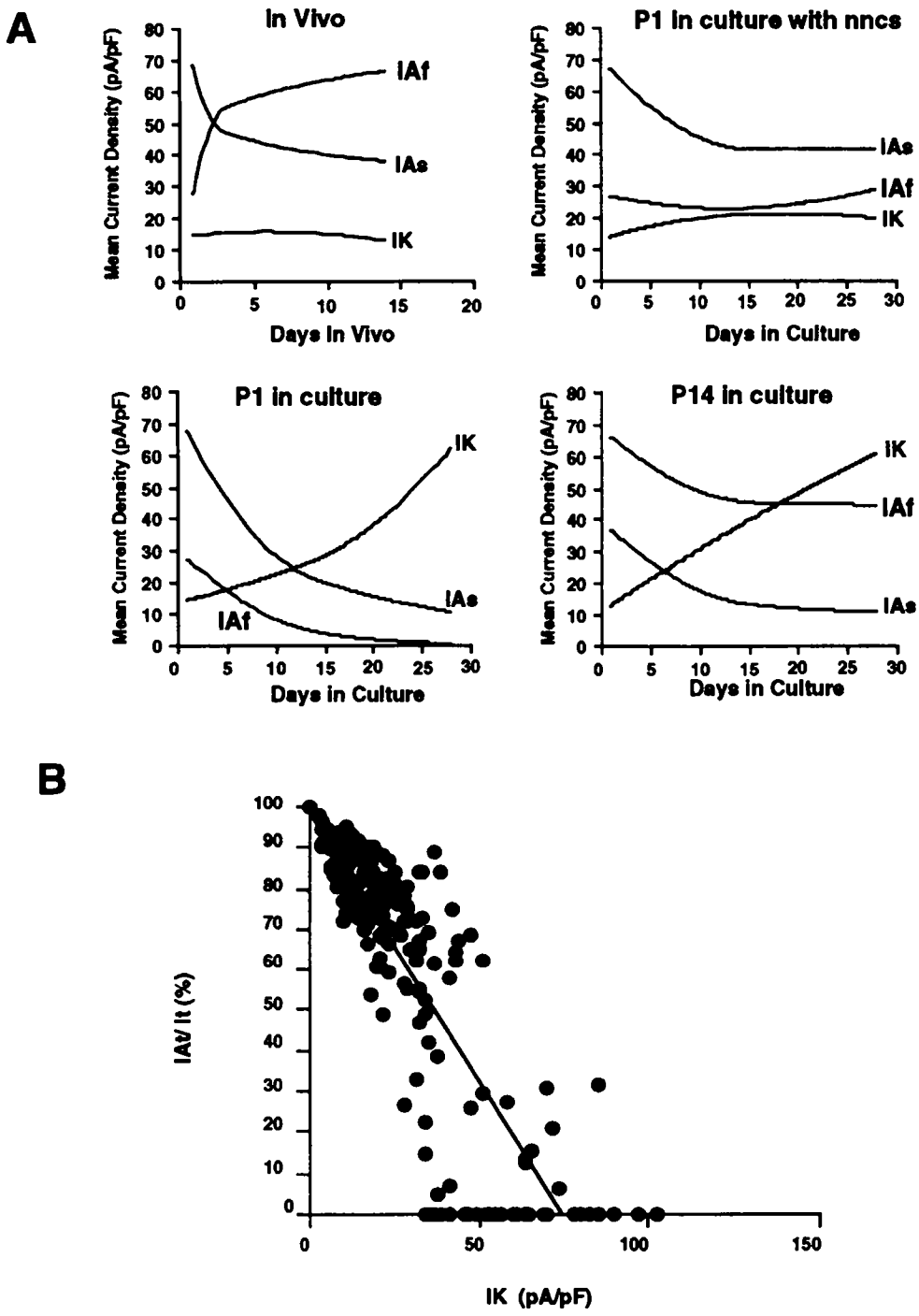

Figure 6. SCG neurons coordinate their expression of I_{Af}, I_{As}, and I_K during postnatal development *in vivo* and in culture. *A* shows schematic diagrams of the changes in I_{Af}, I_{As}, and I_K mean current densities on P1 SCG neurons developing *in vivo* (*top, left*), in culture with their non-neuronal cells (*top, right*), in culture in the absence of their non-neuronal cells (*bottom, left*), and on P14 SCG neurons developing in culture without non-neuronal cells (*bottom, right*). SCG neurons coordinately regulate their expression of the three different voltage-gated K currents such that a decrease in one current is coupled to an increase in one of the other two currents. *B* shows the results of over 250 P1 neurons, either acutely dissociated, grown in culture with or without non-neuronal cells, or grown in the presence of media conditioned by ganglionic non-neuronal cells. For each SCG neuron the size of the A-currents (I_{Af} and I_{As}) as a percentage of the total outward current is plotted against the size of I_K (pA/pF). The *solid line* represents a linear regression with a correlation coefficient (r) of 0.73.

tween I_K density and the contribution of the two A-currents to the outward current. Figure 6*B* plots the A-currents (IAt) as a percentage of the total voltage-gated outward K current (It) versus I_K density (pA/pF) for over 250 P1 SCG neurons from four different conditions, and demonstrates an inverse relationship between I_K densities and I_A densities; we showed a similar relationship for neonatal rat sensory neurons (McFarlane and Cooper, 1991). These results support the idea that there is an intrinsic control mechanism that regulates the appearance of different K channel types in the membranes of peripheral neurons in an interdependent fashion.

Discussion

This article extends our previous investigations on the expression of voltage-gated K currents by neonatal sympathetic neurons in culture. In our earlier study (McFarlane and Cooper, 1992), we showed that I_{Af} and I_{As} on P1 sympathetic neurons decrease significantly over 2–3 weeks when cultured without other cell types. Our results suggest that some factor(s) present *in vivo*, but absent in these cultures, is necessary for the expression of I_{Af} and I_{As}. In this article, we demonstrate that gan-

glionic non-neuronal cells influence the expression of these currents.

Ganglionic non-neuronal cells influence K current expression in culture

When cocultured with ganglionic non-neuronal cells, P1 SCG neurons continue to express I_{Af} and I_{As}. I_{Af} mean current density does not change from P1 levels, and while I_{As} drops by a third over the first 2 weeks in culture, after 28 d I_{As} is still expressed at levels four times greater than on neurons in culture without non-neuronal cells. In addition, I_K is expressed at low levels, similar to that on P1 neurons.

Since non-neuronal cells in our cultures can prevent the loss of I_{Af} and I_{As}, and moreover prevent the increase in I_K, it suggests that the non-neuronal cells are providing some factor(s) that can affect K current expression by SCG neurons. The fact that I_{As} levels are 30% higher in explant cultures than in dissociated cultures might be because I_{As} is more sensitive to the concentration of the non-neuronal cell factor, or alternatively, a ganglionic factor(s), other than non-neuronal cells (Adler and Black, 1985), affects I_{As} expression. The effects of non-neuronal cells on K current expression by P1 SCG neurons are consistent with those of Freschi (1983), who observed small rapidly inactivating currents in SCG neurons cocultured with non-neuronal cells. Several studies have demonstrated that ganglionic non-neuronal cells can influence the differentiated properties of neurons (Furshpan et al., 1982; Nawa and Sah, 1990), including ionic channel expression (Cooper and Lau, 1986; Smith and Kessler, 1988; Clendening and Hume, 1990; Mandelzys and Cooper, 1992). In addition, studies on rat sympathetic neurons developing in culture indicate that neurons grown with non-neuronal cells increase their expression of a Ca-activated K conductance (O'Lague et al., 1978).

Our results suggest that the effects of non-neuronal cells on K current expression are mediated by a secreted factor; we found that media conditioned by non-neuronal cells could mimic the effects of non-neuronal cells in maintaining I_{Af} and I_{As} expression. CNTF has similar effects to those of conditioned media on I_{Af} and I_{As} expression in culture, suggesting that the factor provided by the ganglionic non-neuronal cells could be CNTF. In support of this idea, CNTF is found in high concentrations in the non-neuronal cells of the rat sciatic nerve (Lin et al., 1989), and influences sympathetic neurons to change their transmitter phenotype (Saadat et al., 1989). However, the primary structure of CNTF does not contain an amino-terminal signal sequence, and therefore it is an unresolved question whether CNTF is secreted by the cells in which it is expressed (Lin et al., 1989). In addition to CNTF (Saadat et al., 1989), two other factors have been identified that influence the neurotransmitter phenotype of sympathetic neurons: cholinergic differentiation factor or leukemia inhibitory factor (Yamamari et al., 1989), and a soluble factor from rat sweat glands (Rao and Landis, 1990). It would be interesting to know whether these factors influence the K currents expressed by sympathetic neurons.

There is evidence that second messenger systems may be involved in controlling the expression of K currents on neurons (Desarmenien and Spitzer, 1991). However, given the slow time course for changes in the expression of K currents on neonatal rat SCG neurons, it is unlikely that changes in K current density reflect direct modulation of channel activity by second messengers. It is more likely that transcriptional or translational events control the synthesis of new channels (O'Dowd, 1983; Ribera and Spitzer, 1989). Given the slow time course of changes in K current density, it would be difficult to test this possibility by treating cultures with a transcription inhibitor (Ribera and Spitzer, 1989). A more direct approach will be possible once the K-channel genes expressed by SCG neurons have been identified.

It is probable that factors other than non-neuronal cells are involved in controlling I_{Af} expression. While SCG neurons cocultured with non-neuronal cells continue to express I_{Af} and I_{As}, they do not exhibit the *in vivo* developmental switch from an I_{As} to an I_{Af} dominated outward current (McFarlane and Cooper, 1992). This might imply that when SCG neurons are removed with their non-neuronal cells on P1, I_{Af} expression is arrested at this stage and requires some trigger to allow it to proceed to a more adult stage. We have shown that neither the *in vivo* targets nor the innervating preganglionic spinal neurons provide this trigger. In addition, we can rule out a role for growth factors such as NGF, or CNTF, which when added to the cultures cause none of the developmental changes in K current expression observed *in vivo*. Other factors, possibly hormones, may act as the trigger for changes in K currents; hormones are known to have effects on many of the differentiated properties of neurons (Recio-Pinto et al., 1986; Gould and Butcher, 1989), including their electrical excitability (Desnuelle et al., 1987; Mills and Zakon, 1991; Joels and de Kloet, 1992).

It is possible that a factor from either the preganglionic nerves and/or the target, while not the developmental trigger for I_{Af}, may have a role in regulating the level of I_{Af} expression on SCG neurons. In this regard, P14 SCG neurons, axotomized or denervated on P1, continue to express I_{Af}, but at 20–30% lower levels than control neurons (McFarlane and Cooper, 1992). Interestingly, we observed a similar 30% drop in the mean I_{Af} density on P14 neurons in culture, independent of non-neuronal cells.

Our results indicate that some of the factors influencing I_{Af} expression may have distinct developmental periods during which they can exert their effects. During the first 2 weeks of postnatal development, SCG neurons increase their expression of I_{Af} and decrease their expression of I_{As}, such that by P14 the outward currents appear very similar to those reported for adult SCG neurons (Galvan and Sedlmeir, 1984; Belluzzi et al., 1985). We have found that P14 neurons express I_{Af} when grown in culture without other cell types, in contrast to I_{Af} on P1 neurons, which disappears after 2 weeks under similar culture conditions. These results suggest that the influence that increases I_{Af} expression *in vivo* may also make this current less susceptible to the non-neuronal cell factor; alternatively, I_{Af} may need to be expressed for a certain period of time (2 weeks) before its expression becomes independent of a factor provided by non-neuronal cells. In either case, the first 1–2 weeks of postnatal development appear to be a critical period for I_{Af} during which its expression is increased and stabilized. A similar critical period has been reported for the effect of NGF treatment on the expression of dendrites by SCG neurons (Snider, 1988).

It appears that postnatal SCG neurons do not control their expression of I_{Af} and I_{As} in the same manner. Unlike I_{Af}, I_{As} on P14 neurons retains its P1 dependence on a non-neuronal cell factor; when P14 neurons were cocultured with ganglionic non-neuronal cells almost 70% of the P14 neurons continue to express I_{As}, and over half express I_{As} at densities greater than 30 pA/pF, levels comparable to the mean I_{As} current density on P14 neurons.

Finally, our results suggest that SCG neurons coordinately regulate their expression of I_{Af}, I_{As}, and I_K. For example, we observed that I_K increased over a period that coincided with a decrease in the A-currents. In contrast, if the total A-current level showed little change, the I_K density remained at its *in vivo* level. These results suggest that I_{Af}, I_{As}, and I_K levels on neonatal SCG neurons depend on each other, such that a decrease in the expression of one current leads to a compensatory increase in the expression of the others. In support of this idea, we observed a strong inverse relationship between I_A and I_K current densities such that neurons with high densities of I_{As} and I_{Af} had low I_K densities, whereas neurons with small I_{As} and I_{Af} densities had large I_K densities. This finding is consistent with the hypothesis that neonatal rat SCG neurons have intrinsic control mechanisms to coordinate their control of the expression of different voltage-gated K currents.

References

Adams PR, Galvan M (1986) Voltage-dependent currents of vertebrate neurons and their role in membrane excitability. In: Advances in neurology (Delgado-Escueta AV, Ward AA, Woodbury DM, Porter RJ, eds), pp 137–170. New York: Raven.

Adler JE, Black IB (1985) Sympathetic neuron density differentially regulates transmitter phenotypic expression in culture. Proc Natl Acad Sci USA 82:4296–4300.

Belluzzi O, Sacchi O, Wanke E (1985) A fast transient outward current in the rat sympathetic neurone studied under voltage-clamp conditions. J Physiol (Lond) 358:91–108.

Black IB (1982) Stages of neurotransmitter development in autonomic neurons. Science 215:1198–1204.

Black IB, Mytilineou C (1976) Trans-synaptic regulation of the development of end organ innervation by sympathetic neurons. Brain Res 101:503–521.

Black IB, Hendry IA, Iversen LL (1971) Trans-synaptic regulation of growth and development of adrenergic neurones in a mouse sympathetic ganglion. Brain Res 34:229–240.

Bocchini V, Angeletti PV (1969) The nerve growth factor: purification as an 80,000-molecular-weight protein. Proc Natl Acad Sci USA 64: 787–794.

Bunge R, Johnson M, Ross CD (1978) Nature and nurture in development of the autonomic neuron. Science 199:1409–1416.

Chalazonitis A, Peterson ER, Crain SM (1987) Nerve growth factor regulates the action potential duration of mature sensory neurons. Proc Natl Acad Sci USA 84:289–293.

Clendening B, Hume RI (1990) Cell interactions regulated dendritic morphology and responses to neurotransmitters in embryonic chick sympathetic preganglionic neurons *in vitro*. J Neurosci 10:3992–4005.

Cooper E, Lau M (1986) Factors affecting the expression of acetylcholine receptors on rat sensory neurones in culture. J Physiol (Lond) 377:409–420.

Desarmenien MG, Spitzer NC (1991) Role of calcium and protein kinase C in development of the delayed rectifier potassium current in *Xenopus* spinal neurons. Neuron 7:797–805.

Desnuelle C, Askanas V, Engel WK (1987) Insulin enhances development of functional voltage-dependent Ca^{2+} channels in aneurally cultured human muscle. J Neurochem 49:1113–1118.

Dourado MM, Dryer SE (1992) Changes in the electrical properties of chick ciliary ganglion neurones during embryonic development. J Physiol (Lond) 449:411–428.

Freschi J E (1983) Membrane currents of cultured rat sympathetic neurons under voltage clamp. J Neurophysiol 50:1460–1478.

Furshpan EJ, Potter DD, Landis SC (1982) On the transmitter repertoire of sympathetic neurons in culture. Harvey Lect 76:149–191.

Galvan M, Sedlmeir C (1984) Outward currents in voltage-clamped rat sympathetic neurones. J Physiol (Lond) 356:115–133.

Gould E, Butcher L (1989) Developing cholinergic basal forebrain neurons are sensitive to thyroid hormone. J Neurosci 9:3347–3358.

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.

Joels M, de Kloet ER (1989) Effects of glucocorticoids and norepinephrine on the excitability in the hippocampus. Science 245:1502–1505.

Joels M, de Kloet R (1992) Control of neuronal excitability by corticosteroid hormones. Trends Neurosci 15:25–30.

Landis SC (1990) Target regulation of neurotransmitter phenotype. Trends Neurosci 13:344–350.

Lin LFH, Drzislav M, Lile JD, Lyman GA, Butler ET, Vannice JL, Collins F (1989) Purification, cloning, and expression of ciliary neurotrophic factor (CNTF). Science 246:1023–1025.

Llinas RR (1988) The intrinsic electrophysiological properties of mammalian neurons: insights into central nervous system function. Science 242:1654–1664.

Mandelzys A, Cooper E (1992) Effects of ganglionic satellite cells and NGF on the expression of nicotinic acetylcholine currents by rat sensory neurons. J Neurophysiol 67:1213–1221.

McFarlane S, Cooper E (1991) Kinetics and voltage-dependence of A-type currents on rat sensory neurons in culture. J Neurophysiol 66: 1380–1391.

McFarlane S, Cooper E (1992) Postnatal development of voltage-gated K currents on rat sympathetic neurons. J Neurophysiol 67:1291–1300.

Mills A, Zakon HH (1991) Chronic androgen treatment increases action potential duration in the electric organ of *Sternopygus*. J Neurosci 11:2349–2361.

Nawa H, Sah DW (1990) Different biological activities in conditioned media control the expression of a variety of neuropeptides in cultured sympathetic neurons. Neuron 4:279–287.

Nerbonne JM, Gurney AM (1989) Development of excitable membrane properties in mammalian sympathetic neurons. J Neurosci 9:3272–3286.

O'Dowd DK (1983) RNA synthesis dependence of action potential development in spinal cord neurones. Nature 303:619–621.

O'Lague P, Potter DD, Furshpan EJ (1978) Studies on rat sympathetic neurons developing in culture. I. Growth characteristics and electrophysiological properties. Dev Biol 67:384–403.

Patterson PH (1978) Environmental determination of autonomic neurotransmitter functions. Annu Rev Neurosci 1:1–17.

Patterson PH, Chun LLY (1977) Induction of acetylcholine synthesis in primary cultures of dissociated rat sympathetic neurons. I. Effects of conditioned medium. Dev Biol 56:263–280.

Pragnell M, Snay KJ, Trimmer JS, MacLusky NJ, Naftolin F, Kaczmarek LK, Boyle MB (1990) Estrogen induction of a small, putative K channel mRNA in rat uterus. Neuron 4:807–812.

Rao MS, Landis SC (1990) Characterization of a target-derived neuronal cholinergic differentiation factor. Neuron 5:899–910.

Recio-Pinto E, Rechler MM, Ishii DN (1986) Effects of insulin, insulin-like growth factor-II, and nerve growth factor on neurite formation and survival in cultured sympathetic and sensory neurons. J Neurosci 6:1211–1219.

Ribera AB, Spitzer NC (1989) A critical period of transcription required for differentiation of the action potential of spinal neurons. Neuron 2:1055–1062.

Rudy B (1988) Diversity and ubiquity of K channels. Neuroscience 25:729–749.

Saadat S, Sendtner M, Rohrer H (1989) Ciliary neurotrophic factor induces cholinergic differentiation of rat sympathetic neurons in culture. J Cell Biol 108:1807–1816.

Smith KE, Kessler JA (1988) Non-neuronal cell-conditioned medium regulates muscarinic receptor expression in cultured sympathetic neurons. J Neurosci 8:2406–2413.

Snider WD (1988) Nerve growth factor enhances dendritic arborization of sympathetic ganglion cells in developing mammals. J Neurosci 8:2628–2634.

Thompson SH, Aldrich RW (1980) Membrane potassium channels. In: The cells surface and neuronal function, Vol 6 (Cotman CW, Poste G, Nicholson GL, eds), pp 49–85. Amsterdam: Elsevier.

Wu RL, Barish ME (1992) Two pharmacologically and kinetically distinct transient potassium currents in cultured embryonic mouse hippocampal neurons. J Neurosci 12:2235–2246.

Yamamari T, Fukada K, Aebersold R, Korsching S, Ming-ji F, Patterson PH (1989) The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. Science 246:1412–1416.