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Membrane Depolarization Induces p140^{trk} and NGF Responsiveness, But Not p75^{LNGFR}, in MAH Cells

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Nerve growth factor (NGF) is required for the maturation and survival of sympathetic neurons, but the mechanisms controlling expression of the NGF receptor in developing neuroblasts have not been defined. MAH cells, an immortalized sympathoadrenal progenitor cell line, did not respond to NGF and expressed neither low-affinity NGF receptor (p75) nor p140^{trk} messenger RNAs. Depolarizing concentrations of potassium chloride, but none of a variety of growth factors, induced expression of p140^{trk} but not p75 messenger RNA. A functional response to NGF was acquired by MAH cells under these conditions, suggesting that expression of p75 is not essential for this response. Depolarization also permitted a relatively high proportion of MAH cells to develop and survive as neurons in fibroblast growth factor and NGF. These data establish a relation between electrical activity and neurotrophic factor responsiveness in developing neurons, which may operate in the functioning of the mature nervous system as well.

The survival of vertebrate neurons is dependent on neurotrophic factors secreted by their postsynaptic targets. NGF, the prototypic neurotrophic factor, is required for the survival of sympathetic and some sensory neurons (*1*). The embryonic precursors to sympathetic neurons neither respond to nor require NGF (*2–4*). This raises the question of how developing sympathetic neuroblasts acquire their responsiveness to and dependence on NGF. We have studied this process with the use of MAH cells a retrovirally immortalized sympathoadrenal progenitor cell line (*5*). The identification of the product of the proto-oncogene *trk*, p140^{trk} (Trk), as a signal-transducing subunit of the NGF receptor (NGFR) (*6, 7*) has allowed us to use Trk mRNA expression to assay environmental signals that may induce NGF responsiveness in MAH cells. Here we identify membrane depolarization as one such signal.

MAH cells, like the nonimmortalized progenitors from which they derive, do not undergo neuronal differentiation in response to NGF. The protein p75, the low-affinity NGFR (*8, 9*), is not expressed by these cells (*5*). MAH cells grown in the absence of added factors also express little or no Trk mRNA (Fig. 1A, lanes 1 and 2). Thus, the failure of these precursor cells to respond to NGF correlates with their lack of expression of both types of NGFR mRNAs. We then sought to identify factors that induce expression of NGFR and NGF responsiveness. Previously, we found that basic fibroblast growth factor (bFGF) induced low levels of p75 expression and NGF responsiveness in a small subpopulation of MAH cells (*5*). However, bFGF failed to induce significant Trk expression in MAH cells, as did a number of other growth and neurotrophic factors (Fig. 1A, lanes 4 through 7, and data not shown). In addition, retinoic acid, which induces high-affinity NGF receptors and NGF dependence in chick sympathetic neuroblasts (*10*), did not induce Trk mRNA (Fig. 1A, lane 8).

In the chick, depolarization increases the survival of NGF-dependent sympathetic neurons (*4*). In MAH cells, depolarization stimulated the survival of postmitotic neurons. Depolarization of MAH cells produced by the addition of 40 mM KCl led to an induction of Trk mRNA (Fig. 1A, lane 3). A time course in 40 mM KCl revealed that Trk expression was detectable within 24 hours and reached maximal amounts within 3 days (Fig. 1C, lanes 5 through 8). Reprobing of the same blots with p75 probes revealed that, in contrast to Trk mRNA, p75 mRNA was not induced by 40 mM KCl.

MAH cells require dexamethasone (dex) for long-term survival; when dex is removed, the cells die within 4 to 5 days. In the presence of 5 μM dex, a low steady-state amount of Trk mRNA was detected (Fig. 1B, lane 2). However, even in the presence of dex an up-regulation of Trk mRNA by 40 mM KCl occurred (Fig. 1B, lane 3), indicating that the effect of depolarization is not simply to maintain the survival of Trk-expressing MAH cells. The time course of Trk induction by 40 mM KCl was similar in the presence of dex (Fig. 1D, lanes 6 through 9) as in its absence, although higher steady-state amounts of Trk mRNA were produced in the presence of dex (compare Fig. 1D, lane 8, with Fig. 1C, lane 7). As was the case in the absence of dex, no induction of p75 mRNA was detected in 40 mM KCl plus dex.

The effect of 40 mM KCl is likely to be produced by membrane depolarization because no induction of Trk mRNA was observed in 40 mM NaCl (Fig. 1, A and B, lanes 9). Moreover, veratridine, an Na^+ channel agonist that leads to membrane depolarization, also caused an increase in the amount of Trk mRNA concentrations (data not shown). In PC12 cells, the induction of immediate-early gene expression by membrane depolarization requires the opening of voltage-gated Ca^{2+} channels and depends on extracellular Ca^{2+} (*11*). Removal of extracellular Ca^{2+} or addition of dihydropyridine antagonists of voltage-gated Ca^{2+} channels resulted in the death of MAH cells within 24 hours, precluding our ability to determine a requirement for Ca^{2+} influx in Trk induction. However, at suboptimal concentrations of KCl (20 mM) (Fig. 1E, lane 4), the Ca^{2+} channel agonist Bay K 8644 potentiated the induction of Trk mRNA (Fig. 1F, lanes 3 and 4), which suggests that Ca^{2+} influx through voltage-gated L-type Ca^{2+} channels is indeed involved in the induction of Trk mRNA by membrane depolarization.

We then sought to determine whether depolarization induces a functional response to NGF. We used two assays of NGF responsiveness: neurite outgrowth and cell number. Cell number reflects both the survival- and proliferation-promoting (*12*) effects of NGF, although for technical reasons it is difficult to determine the relative contributions of these two processes in this system. NGF responses were assayed after 5 days, by which time most MAH cells had died in control medium (Table 1). Those few cells that survived showed little process outgrowth (Fig. 2A). Similar results were obtained in NGF alone (Fig. 2B and Table 1), indicating that MAH cells do not respond to this factor. Cell number was significantly increased by depolarizing concentrations of KCl (Table 1), although little neurite outgrowth was observed (Fig. 2C). In NGF plus 40 mM KCl, cell number was even higher (Table 1) and the cells bore long neurites (Fig. 2D). These neurite-bearing cells, however, lacked the cell soma hypertrophy characteristic of mature neu-

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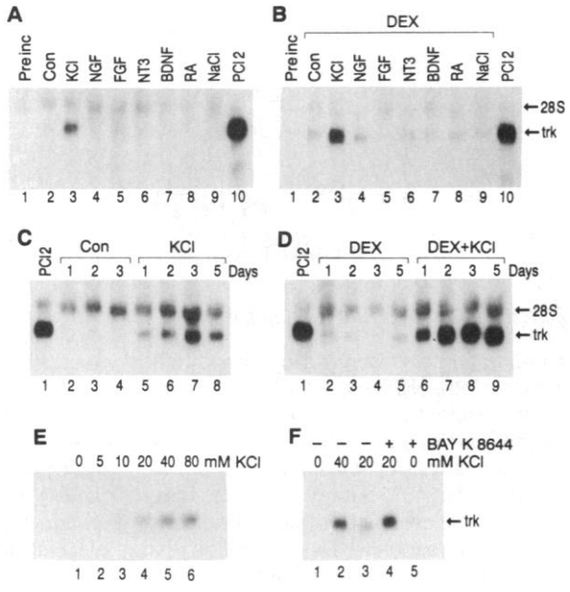

Fig. 1. Effect of elevated K^+ on Trk mRNA expression in MAH cells. A series of Northern (RNA) blots containing 5 μg per lane of total RNA, probed with a ^{32}P-labeled rat Trk cRNA probe derived from the plasmid pDM97 (*29*). Equal loading of RNA samples was verified by ethidium bromide staining of ribosomal RNA (rRNA) or by hybridization with a γ-actin probe (data not shown). MAH cells were preincubated in the absence of factors for 24 hours. Various factors were then added and RNA isolated after a further 3 days of incubation, except where indicated. (**A**) Lane 1, preincubated cells; lane 2, control; lane 3, 40 mM KCl; lane 4, 50 ng of NGF per milliliter; lane 5, 10 ng of FGF per milliliter; lane 6, 25 ng of recombinant neurotrophin-3 (NT-3) per milliliter; lane 7, 25 ng of recombinant brain-derived neurotrophic factor (BDNF) per milliliter; lane 8, 5 × 10^{-9} M retinoic acid; lane 9, 40 mM NaCl; lane 10, PC12 cell RNA. (**B**) The same conditions as (A), except that after the preincubation all factor additions were performed in the presence of 5 μM dex. (**C**) Time course of KCl-induced Trk expression. MAH cells were plated in the absence (lanes 2 to 4) or presence (lanes 5 to 8) of 40 mM KCl (without preincubation), and RNA was isolated after 1, 2, 3, or 5 days. Lane 1, PC12 cell RNA. (**D**) MAH cells were plated in 5 μM dex in the absence (lanes 2 to 5) or presence (lanes 6 to 9) of 40 mM KCl. RNA was isolated after 1, 2, 3, and 5 days. Lane 1, PC12 cell RNA. (**E**) Dose response of Trk induction by KCl. MAH cells were treated with 0, 5, 10, 20, 40, or 80 mM KCl (lanes 1 through 6, respectively) for 3 days. (**F**) The Ca^{2+}-channel agonist BAY K 8644 potentiates Trk induction by KCl. Cells were treated with 20 mM KCl in the presence (lanes 4 and 5) or absence (lanes 1 to 3) of 1 μM BAY K 8644 for 3 days. Lane 1, control; lane 2, 40 mM KCl; lane 3, 20 mM KCl; lane 4, 20 mM KCl plus 1 μM BAY K 8644; lane 5, 1 μM BAY K 8644 alone. The *trk* transcript is indicated by an arrow; "28*S*" indicates cross-hybridization of probe to rRNA. Similar results were obtained in at least two or more independent experiments. Reprobing of the same or similar blots with a p75 cRNA probe yielded a strong signal in PC12-positive control lanes but no signal in MAH cells under any of the conditions tested (data not shown).

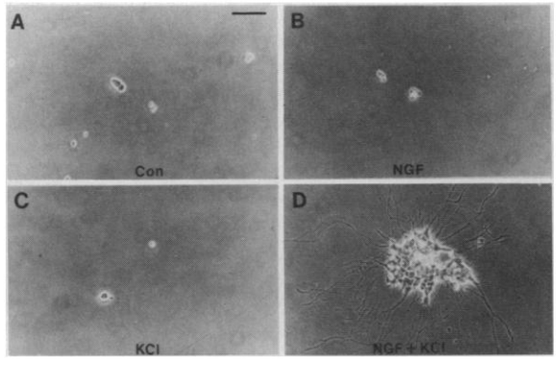

Fig. 2. Effect of depolarization on the functional response to NGF in MAH cells. Cells plated on a poly-D-lysine plus laminin substrate in control medium (**A**), in 50 ng of NGF per milliliter (**B**), in 40 mM KCl (**C**), or in NGF plus KCl (**D**) were photographed after 5 days of culture. Quantitation is shown in Table 1. Surviving cells in (D) constituted 20 to 25% of the maximum number of cells measured on days 1 to 3. Bar in (A), 85 μm.

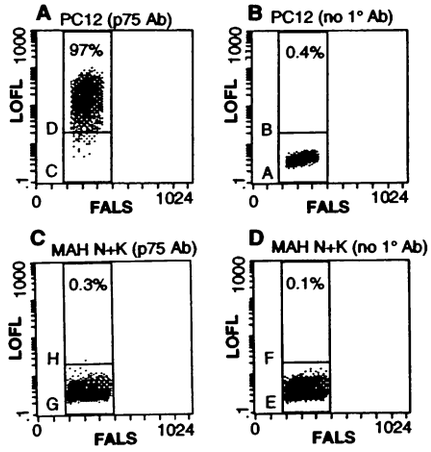

Fig. 3. MAH cells grown in NGF plus 40 mM KCl do not express low-affinity NGF receptor (p75). (**C** and **D**) MAH cells were grown in NGF plus KCl as in Fig. 2, harvested after 5 days, and surface-labeled with monoclonal antibody 192-Ig (anti-p75) followed by fluorescein-conjugated goat antibody to mouse immunoglobulin G (TAGO, Inc., Burlingame, California). (**A** and **B**) PC12 cells were analyzed in parallel as a positive control. (B) and (D) contained no primary antibody. Flow cytometry was performed on a Coulter Elite instrument. LOFL, log of fluorescence. FALS (forward angle light scatter) is a measure of cell size. The percentage of p75-positive cells in the population is shown in the gated region. No significant staining of MAH cells (C) is observed above control (D), whereas PC12 cells (A) are strongly positive for p75.

rons and resembled a previously described immature neuroblast state in this lineage (*3*). Thus, depolarization induced not only expression of Trk mRNA but also at least a short-term functional response to NGF, suggesting that Trk mediates this response. However, even in NGF plus 40 mM KCl, only about 20 to 25% of MAH cells survived to day 5, suggesting that not all cells expressed sufficient Trk or that Trk and NGF are insufficient to support the survival of all cells under these culture conditions.

The finding that depolarization induces Trk mRNA and NGF responsiveness in MAH cells, but does not induce p75 mRNA, implies that p75 is not required in MAH cells for at least short-term NGF responsiveness. However, because Trk mRNA and p75 mRNA expression were assayed on day 3 and functional responsiveness to NGF was assayed on day 5, it is possible that MAH cells had acquired p75 expression by day 5. To resolve this issue, we stained MAH cells grown for 5 days in NGF plus KCl with a monoclonal antibody to p75, 192-Ig (*13*), and analyzed the cells by flow cytometry. PC12 cells, which express p75, were analyzed in parallel as a positive control. Whereas over 97% of the PC12 cells were strongly labeled by the 192-Ig antibody (Fig. 3A), the NGF plus KCl-treated MAH cells were not detectably labeled (Fig. 3C). These results were confirmed by immunohistochemical analysis of process-bearing MAH cells grown in NGF plus KCl. These cells clearly showed a functional response to NGF (survival and neurite outgrowth) but failed to express p75 as determined by a sensitive nickel-diaminobenzidine immunostaining method. These data suggest that in MAH cells, Trk is sufficient to mediate a biological response to NGF in the absence of detectable p75 protein.

Previously, we found that growth of MAH cells in FGF plus NGF allows a small proportion of the cells to survive as NGF-dependent, postmitotic neurons (*5*). The inefficiency of such neuronal survival has limited the utility of MAH cells as a model system for studying neuronal differentiation. Because elevated potassium has been shown to promote the survival of both immature chick sympathetic neuroblasts (*4*) and postnatal rat sympathetic neurons (*14*), we reasoned that such a depolarizing

Table 1. MAH cells grown in depolarizing conditions display a functional response to NGF.

Treatment	Percentage of process-bearing clusters*	Cell number*
Control	2 ± 1	71 ± 26
KCl†	7 ± 2	716 ± 58
NGF‡	2 ± 1	47 ± 10
NGF+KCl	45 ± 4	2162 ± 87

*Two thousand cells were initially plated per well in 24-well dishes. The percentage of cell clusters bearing processes, and total cell number, were determined after 5 days in culture. Values are the mean ± SEM of triplicate determinations from a representative experiment. Similar results were obtained in a minimum of three independent experiments. †40 mM KCl. ‡50 ng of NGF per milliliter.

treatment might similarly enhance the long-term survival of MAH cell–derived neurons. To test this idea, we compared the number of MAH cells that developed and survived as neurons after 10 days in FGF plus NGF, with or without 40 mM KCl. We observed that many more neurons exhibiting characteristic cell soma hypertrophy and distinctive nucleoli survived in the presence of 40 mM KCl (Fig. 4B) than in its absence (Fig. 4A). In one representative experiment, 2000 MAH cells per well were initially plated and yielded 261 ± 52 neurons per well (mean ± SEM, n = 5) in NGF plus FGF, versus 1324 ± 315 neurons per well in NGF plus FGF plus KCl, a fivefold enhancement. It is difficult to calculate the efficiency of survival because of the cell proliferation that occurs between plating and the time of assay. In the presence of KCl, the survival of most neurons was independent of NGF, as demonstrated for primary sympathetic neurons (*14*).

These data suggest a possible relation between electrical activity and trophic factor responsiveness in developing neurons. In the rat, developing sympathetic ganglia become innervated between embryonic day 12 (E12) and E13 (*15*, *16*). Growing sympathetic axons reach their peripheral targets several days later at E15 (*17*) and then acquire NGF dependence. The relative timing of these events is consistent with a role for preganglionic activity in the induction of Trk. In support of this hypothesis, blocking preganglionic cholinergic transmission has been shown to decrease survival of chick sympathetic neurons (*18*). Such a mechanism is attractive because it could provide a way to coordinate the formation of stable preganglionic and postganglionic synapses. However, we do not know whether the extent or duration of depolarization required for Trk induction in vitro is normally achieved in vivo.

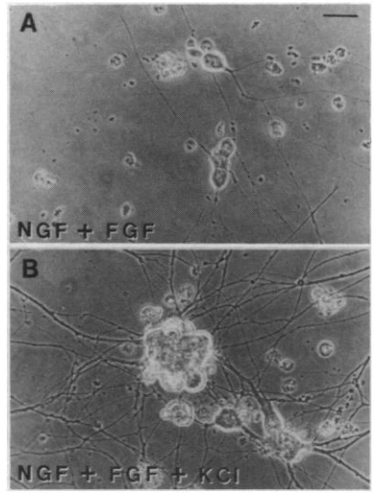

Fig. 4. Effect of depolarization on the number of neurons that develop from MAH cells grown in FGF plus NGF. Representative fields are shown from MAH cell cultures grown for 10 days in either 50 ng of NGF per milliliter plus 10 ng of FGF per milliliter (**A**) or NGF plus FGF plus 40 mM KCl (**B**). Cells were fed after 4 days and replated after 7 days. Quantification of a representative experiment in which 2000 MAH cells per well were initially plated yielded 261 ± 52 neurons per well (mean ± SEM, n = 5) in NGF plus FGF, and 1324 ± 315 neurons per well in NGF plus FGF plus KCl, a fivefold difference. Bar in (A), 32 μm.

In order to survive, developing neuroblasts must both express NGF receptors and extend axons to reach peripheral sources of NGF (*19*, *20*). These two aspects of neuronal development are independently controlled, as illustrated by the observation that chromaffin cells are NGF-responsive but lack axons (*21*, *22*). We have identified candidate environmental signals that control these distinct processes. Membrane depolarization induces expression of Trk but does not cause neurite outgrowth. Conversely, bFGF promotes neurite outgrowth (*23*) but does not induce Trk mRNA. (However, after long-term exposure to FGF and NGF, a small proportion of MAH cells become NGF-dependent (*5*); it is not yet clear whether or how such extended FGF treatment causes expression of Trk.) Depolarization is not the only signal that can induce Trk mRNA; dibutyryl cyclic adenosine 3′,5′-monophosphate (cAMP) has a similar effect (*24*), suggesting that agents that activate adenylate cyclase may induce Trk as well. It remains to be determined which signals are most important for Trk expression in vivo.

Our data indicate that developing neurons expressing Trk but not p75 display a functional response to NGF (although we cannot exclude low-level expression of p75 below the detection limits of our assays). This result is consistent with the observations that PC12 cells are able to respond to NGF in the presence of a blocking antibody to p75 (*25*), that *Xenopus* oocytes and fibroblasts expressing Trk but not p75 respond to NGF (*26*, *27*), and that a mutant NGF lacking a p75 binding site retains biological activity on PC12 cells (*28*). Nevertheless, it has been shown that coexpression of p75 with Trk increases NGFR affinity in fibroblasts (*29*). Although these observations are difficult to reconcile, MAH cells should provide a neuronal system for analyzing the relative contributions of p75 and Trk to NGFR function and identifying the signals that control expression of these receptors during development.

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