

Retinoic Acid Combined with Neurotrophin-3 Enhances the Survival and Neurite Outgrowth of Embryonic Sympathetic Neurons

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Both nerve growth factor (NGF) and neurotrophin-3 (NT-3) are necessary for the survival of embryonic sympathetic neurons *in vivo*. All-*trans* retinoic acid (atRA) has been shown to promote neurite outgrowth and long-term survival of chick embryonic sympathetic neurons cultured in the presence of NGF. The present study shows that atRA can also potentiate the survival and neurite outgrowth-promoting activities of NT-3. This was accomplished by enhancing the survival of existing neurons, as cell proliferation was unaffected by exposure to atRA. atRA also enhanced neurite outgrowth of the NT-3-treated cells; however, the neurites appeared thicker and less branched than cells treated with atRA in combination with NGF. Using a quantitative PCR assay, *trkA* and *p75^{NTR}* mRNAs, but not *trkC* mRNA, were increased (~1.5- to 2-fold) after 72 and 48 hr of exposure of the cultures to atRA, respectively. The atRA-induced increase in *trkA* mRNA may play a role in the enhanced survival of neurons cultured in the presence of either NGF or NT-3, as both neurotrophins have been shown to signal through this receptor. The time course of these mRNA changes would indicate that atRA does not regulate the neurotrophin receptor mRNA directly, rather, intervening gene transcription is required. Thus, during development, atRA may play a role in fine-tuning embryonic responsiveness to both NT-3 and NGF.

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The number of neurons initially produced in the developing sympathetic nervous system exceeds the number that ultimately survive in the mature nervous system. The survival of sympathetic neurons is determined, at least in part, by the presence of limiting amounts of neurotrophic factors (1, 2). Nerve growth factor (NGF) was the first neurotrophic factor to be discovered and it serves as a survival factor for developing sympathetic neurons (1). Mice null for the NGF gene exhibit a complete loss of neurons in the developing superior cervical ganglia (SCG) (3). A role for neurotrophin-3 (NT-3) in these neurons has also been described (4-6). Targeted deletion of NT-3 results in the death of 50% of this neuronal population (4, 7-8). Cell culture experiments have indicated that NT-3 plays an important role early in the survival of sympathetic neuroblasts (4-6), whereas gene knockout studies in mice have provided evidence for both early and late developmental effects (4, 8, 9). NT-3 mRNA has been detected in both non-neuronal cells early in the development of the SCG as well as in sympathetic targets (4, 10, 11). Thus, it is clear that both NGF and NT-3 are needed by the developing sympathetic nervous system.

Small diffusable factors such as the vitamin A-metabolite, all-*trans* retinoic acid (atRA), have also been shown to play a role in the survival and differentiation of the developing sympathetic nervous system. atRA is essential for the long-term survival of embryonic chick sympathetic neurons cultured in the presence of NGF (12, 13). Not only is atRA essential for early sympathetic neuronal survival in culture, but it has also been shown to enhance neurite outgrowth in NGF-treated cultures at later stages of development and it is important for the maintenance of these neurites (13). Although it is clear that atRA participates with NGF in promoting the survival and differentiation of sympathetic neurons, it is unknown whether neuronal responsiveness to NT-3 is influenced by retinoid.

The mechanisms whereby atRA and neurotrophins ex-

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ert their activities are quite different. atRA binds to nuclear receptors (RARs) that belong to the steroid/thyroid superfamily of receptors (14), whereas neurotrophins bind to integral membrane receptors. RARs function as ligand-activated transcription factors that can either bind to specific retinoic acid response elements (RARE) in the promoter region of regulated genes to modulate their expression, or alternatively, they may alter the function of other transcription factors via protein-protein interactions. atRA has been shown to be present in early chick embryos, although it is unknown whether it can be produced by cells within the developing sympathetic ganglia (15–20). Retinoid receptors have also been identified in developing embryonic sympathetic ganglia (13, 21). Thus, retinoid ligand and receptors are present at a time consistent with a role in normal development of the sympathetic nervous system.

Neurotrophins act by binding to receptors containing an intracellular tyrosine kinase domain, known as trks (22). TrkA is a high-affinity receptor for NGF (22) and low levels of *trkA* mRNA are found early in the development of embryonic sympathetic neurons in the chick (23). Steady-state levels of *trkA* mRNA increase with development in the lumbosacral sympathetic ganglia of the chick, coincident with increasing responsiveness of these neurons to NGF (13). Mice lacking *trkA* exhibit a complete loss of sympathetic neurons, emphasizing the importance of this receptor in the development of this neuronal population (24). NT-3 has been shown to bind to trkC with high affinity (22) and *trkC* mRNA is also present in young sympathetic neurons (4, 8, 25). As the embryo develops, the expression of *trkC* mRNA diminishes, whereas the mRNA for *trkA* increases. However, the necessity for trkC to mediate NT-3 responsiveness in developing murine sympathetic neurons has recently been challenged, as the targeted deletion of *trkC* does not affect the survival of this population of neurons (7, 25).

The neurotrophins, NT-3, brain-derived neurotrophic factor (BDNF), and NGF also bind p75^{NTR}, a cell surface receptor that is a part of the tumor necrosis receptor family (26). p75^{NTR} binds all three neurotrophins with equal affinity (22). Although the biological function of p75^{NTR} is not completely understood, a number of experiments suggest that it plays a role in neuronal survival (26, 27). It has been suggested that p75^{NTR} suppresses responsiveness to NT-3 (28, 29).

The mechanism whereby atRA influences the survival and differentiation of embryonic sympathetic neurons in the presence of the neurotrophin, NGF, is unclear, although an effect on the expression of neurotrophin receptors has been proposed. One group reported that when immature (non-NGF responsive) sympathetic neurons are explanted and cultured in the presence of atRA, *trkA* mRNA is rapidly induced, resulting in the appearance of high-affinity NGF-binding sites (21). However, other groups have shown that *trkA* mRNA is either repressed or unchanged in the presence of atRA (23, 30–32).

The objectives of the present study were to determine

whether atRA could potentiate the effects of NT-3 in developing embryonic sympathetic neurons, and if so, at what developmental stages neurons were most affected. Secondly, we sought to determine whether atRA could influence the expression of mRNAs encoding for neurotrophin receptors that have been proposed to play a role in the developing sympathetic nervous system, including *trkA*, *trkC*, and p75^{NTR}.

Materials and Methods

Materials. atRA was purchased from Eastman Kodak (Rochester, NY). The purity of atRA (>99%) was verified by reverse-phase HPLC (33). Purified mouse NGF (2.5S) was obtained from Promega (Madison, WI) and recombinant human NT-3 and BDNF were generously provided by Regeneron Pharmaceuticals, Inc. (Tarrytown, NY). The monoclonal antibody to mouse neurofilament-M was kindly provided by Dr. Virginia Lee (University of Pennsylvania Medical School, Philadelphia, PA) and the antineuron nuclear primary antiserum was graciously provided by Dr. Miles Epstein (University of Wisconsin, Madison, WI).

Neuronal Culture. Sympathetic neurons were prepared from chick embryos and were cultured as described (13). Briefly, sympathetic ganglia (lumbosacral) were dissected from chick embryos at various embryonic stages (stages 30 and 31 [E6.5], 37 [E11], or 40 [E14]) (34). Ganglia were incubated in the presence of trypsin and were triturated to yield a single-cell suspension. The cells were preplated on tissue culture dishes to remove non-neuronal cells. Neurons (20,000 or 100,000) were then placed in poly-DL-ornithine- and laminin-coated dishes (24-well plates or 35-mm dishes) and were cultured for either 7 days (cell survival and neurite outgrowth) or 4 to 72 hr (RNA isolation).

Neuronal Staining. Cells were stained and photographed as described previously (13). After 7 days in culture, sympathetic neurons were fixed with 2% paraformaldehyde. Neuronal cell bodies were identified using a human antiserum that recognizes a neuronal nuclear antigen (35). Neurites and cell soma were stained using a monoclonal antibody directed against neurofilament-M (36).

Cell Proliferation Assay. Cultures of sympathetic neurons from E6.5–7 and E11 chick embryos were prepared as described above. Five hours after plating and dosing the cells with neurotrophin or neurotrophin and atRA, 5-bromo-2'-deoxyuridine (BrDU; Cell Proliferation Kit, Amersham Pharmacia Biotech Inc., Piscataway, NJ) was added. Twelve hours after addition of BrDU, the cells were fixed and the incorporated BrDU was detected immunocytochemically using an antibody to BrDU, followed by a secondary peroxidase conjugated antibody to mouse immunoglobulin (IgG2a) and incubation with the substrate diaminobenzidine. Duplicate wells were prepared for each treatment group. The percentage of cells incorporating BrDU was determined by counting 12 random fields in each

well. Each culture experiment was repeated two to three times.

Quantitative PCR (Q-PCR) Assay. The Q-PCR assay was developed based on the method described by Estus *et al.* (37). Cultures were exposed to NGF or NGF + atRA and mRNA was isolated from neurons using oligo(dT)-cellulose (QuickPrep *Micro* mRNA Purification Kit, Pharmacia Biotech Inc.). The mRNA was reverse transcribed in a final volume of 25 μ l using 15 units AMV reverse transcriptase, 100 pmol random hexamers, 28 units of RNasin ribonuclease inhibitor, AMV reverse transcriptase reaction buffer (50 mM Tris-HCl, pH 8.3, 25°C, 50 mM KCl, 10 mM MgCl₂, 0.5 mM spermidine, and 10 mM dithiothreitol (DTT) and 0.4 mM each dATP, dCTP, dGTP, and dTTP. The RNA and random hexamers were heated at 65° to 70°C for 3 min prior to the addition of the other reaction components. The reactions were then incubated at 20°C for 10 min followed by incubation at 42°C for 50 min, dilution with 175 μ l of sterile water and heat treatment for 5 min at 90°C. The reverse transcribed material (cRNA) was then determined to be devoid of genomic DNA by amplifying in the presence of β -actin primers known to span an intron (38). The volume of sample containing equivalent amounts of cRNA was determined by PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or L27^{osp} ribosomal cDNA. The volumes from each treatment group that generated equivalent amounts of GAPDH or L27 product were used in the analysis of target genes (*trkA*, *trkC*, p75^{NTR}, and RAR β 2). All PCRs were prepared in a final reaction volume of 50 μ l using 1.5 mM MgCl₂, 0.1 mM (each) dATP, dGTP, and dTTP, 0.05 mM dCTP, 0.3 μ M primers (GAPDH [486–1043]: upstream = ATT GTC AGC AAT GCA TCG TGC ACC; downstream = CAT GTG GAC CAT CAA GTC CAC AAC [39]; L27 [92–218]: upstream = GGC TGT CAT CGT GAA GAA CAT C; downstream = CTT CGC TAT CTT CTT GCC C [40]; *trkA* [1594–1737 from GenBank accession no. U43396]: upstream = ACC ACG TGC AGC GCC GCG ACA TTG TGC TC; downstream = GGC TCA CCC TCG GTG CAC ACG CCA TAG AA; *trkC* [1422–1592 from GenBank accession no. S74248] upstream = GAT TGT GGC CAC CAA CCA; downstream = ACC CCA AAT GTG TCC TCC [41]; p75^{NTR}[1138–1316]: upstream = ACG TTT CTA CGT CTG GCG; downstream = AGC GTG CAT GTG TGC ATT [42]; RAR β [94–262]: upstream = TTG CAG GCA TTC TGT ACA GG; downstream = GGT GTT GCC ACT CTG TTT GT [43]), 15 μ Ci [α -³²P]dCTP, *Taq* DNA polymerase reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0 at 25°C, 0.1% Triton X-100), and 2 units of *Taq* DNA polymerase. Each reaction was overlaid with 30 μ l of mineral oil and was amplified in a Perkin-Elmer 480 DNA thermal cycler using the following conditions: denaturation for 1 min at 94°C, annealing for 2 min at 60°C, and extension for 3 min at 72°C. Each set of reactions was done in triplicate. In addition, all reactions were amplified for a minimum of three different cycle numbers to verify

that products were within the linear range of amplification. Every target gene at every time point was measured from a minimum of three independent cell culture experiments. A fraction (10 μ l) of each reaction mixture was analyzed using polyacrylamide gels (5%–6%). The gels were dried, exposed to a phosphor screen, and the amount of radiolabeled product was quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis. Statistical analysis was performed with the assistance of the University of Wisconsin-Madison CALS Statistical Consulting Group. All analyses were done with SAS software using an analysis of variance (ANOVA) followed by pairwise comparisons using Bonferroni and least significant difference *t* tests. Significance was set at *P* < 0.05.

Results

atRA Enhances the Survival of Embryonic Sympathetic Neurons Cultured in the Presence of Either NT-3 or NGF, But Not BDNF. The effect of atRA on survival was analyzed in embryonic sympathetic neurons after they had been cultured with NT-3, NGF, or BDNF for 7 days. Neurons were dissected and studied at three different stages of chick development (E6.5-7, 11, and 14). The earliest stage studied (E6.5-7) is shortly after gangliogenesis has begun in the chick embryo (44–46). E11 is the time when sympathetic neurons begin to innervate their target tissues, and by E14, the majority of neurons are NGF dependent (47, 48). The results in Figure 1 and Tables I and II show that long-term (7 day) survival was significantly increased after the exposure of neurons to both NT-3 (20 ng/ml) and atRA at all three developmental stages when compared with cultures treated with either NT-3 or atRA alone. At E6.5-7, NT-3 in combination with atRA was equally effective in increasing neuronal survival when tested at both 2 and 20 ng/ml (data not shown). The responsiveness of sympathetic neurons to NT-3 was somewhat diminished at E14 compared with neurons explanted at earlier times, regardless of whether atRA was present.

The combination of NGF and atRA also increased the number of surviving neurons at all three developmental stages when compared with survival rates in the presence of either NGF or atRA alone. The percentage of neurons that survived in the presence of both NT-3 and atRA was always less than that observed in the presence of both NGF and atRA. In addition, the number of cells surviving in the presence of NGF + NT-3 + atRA was not additive or synergistic, indicating that both neurotrophins were acting on the same population of cells. Thus, atRA enhanced the overall survival-promoting effect of NT-3, but not to the level seen in the presence of both atRA and NGF.

In order to establish the specificity of the atRA effect on neurotrophin responsiveness, studies were also conducted with BDNF. BDNF was chosen because *in vitro* studies have indicated that BDNF does not support survival or neurite outgrowth of sympathetic neurons (49, 50). Fur-

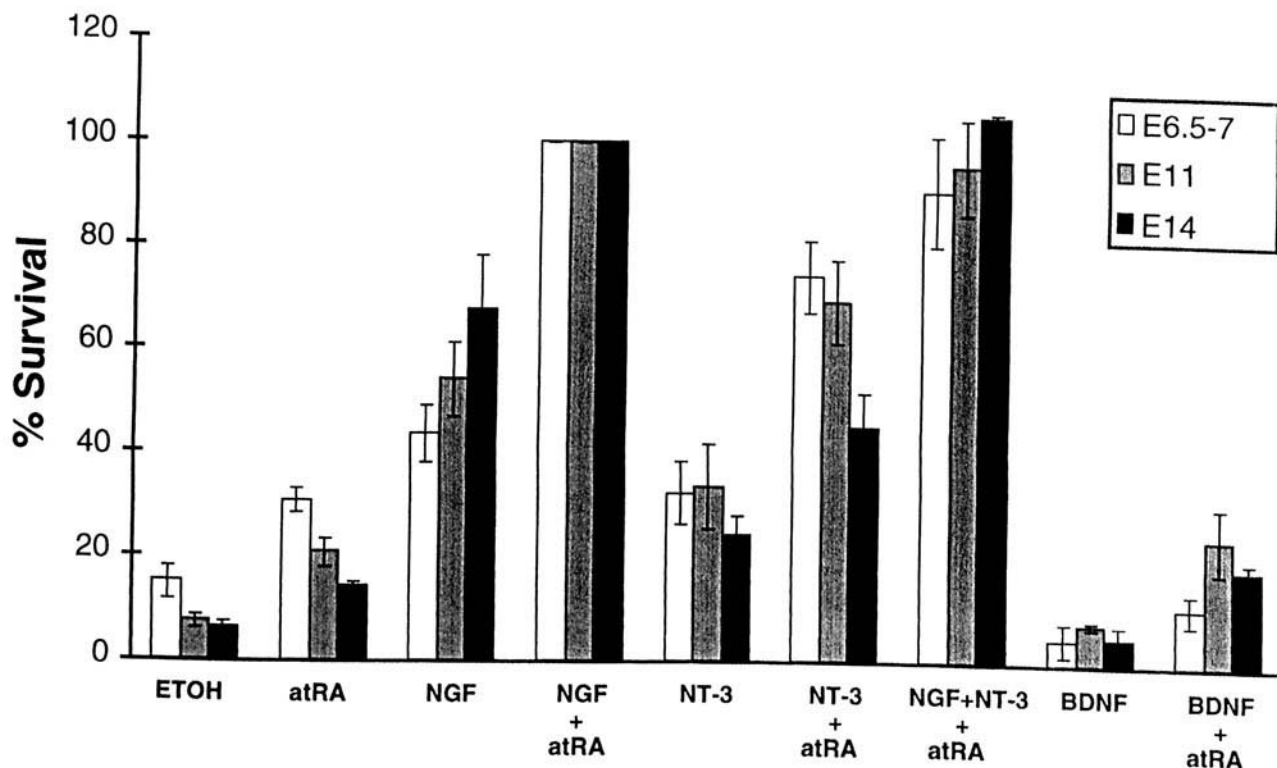


Figure 1. atRA enhances the survival of embryonic sympathetic neurons in the presence of NGF or NT-3, but not BDNF. Primary cultures of sympathetic neurons were prepared from chick embryos at three different stages of development (E6.5-7, E11, and E14). The cells were treated with either 0.2% ETOH, 5×10^{-9} M atRA, 20 ng/ml neurotrophin, or a combination thereof. After 7 days in culture, survival was determined by counting 24 random fields of cells that had been treated with fluorescein diacetate. Survival was expressed as a percentage of the number of surviving neurons present in the NGF + atRA group. Three to nine independent culture experiments (e.g., the dissection and culture experiment were performed on separate occasions) were done for each developmental time point. Statistical analysis was performed using ANOVA followed by pairwise comparisons using Bonferroni and least significant difference *t* tests with significance set at $P < 0.05$. The letters indicate the statistically significant differences when comparisons are made across the three developmental stages. Those groups with the same letter differ significantly. The complete statistical analyses across treatment and within a developmental stage are summarized in Tables I and II. Values shown are mean \pm SE.

Table I. Statistical Analysis of Survival Differences* Due to Treatment Within a Developmental Stage

| Stage | ETOH | atRA | NGF | NGF + atRA | NT-3 | NT-3 + atRA | NGF + NT-3 + atRA | BDNF | BDNF + atRA |
|--------|-------------|---------|-----------------|---------------|-------------|-----------------|-------------------|---------------|---------------|
| E6.5-7 | a,b,c,d | e,f,g,h | b,i,j,k,l,m | c,g,l,n,o,p,q | p,r,s,t | d,h,m,q,t,u,v,w | a,f,k,s,w,x,y | e,i,n,r,u,x | o,v,y |
| E11 | a,b,c,d,e,f | g,h,i,j | c,h,k,l,m,n,o,p | d,i,n,q,r,s,t | e,o,s,u,v,w | f,j,p,t,w,x,y,z | b,g,m,v,z,aa,bb | k,q,u,x,aa,cc | a,l,r,y,bb,cc |
| E14 | a,b,c,d,e | f,g,h,i | b,g,j,k,l,m,n,o | c,h,m,p,q,r,s | d,n,r,t,u | e,i,o,s,u,v,w,x | a,f,j,t,x,y,z | j,p,v,y | k,q,w,z |

Note. Only horizontally read comparisons can be made in this table, i.e., treatment groups cannot be compared across stages. * Treatments within a given stage with the same letter differ significantly. For example, at E6.5-7, NGF is significantly different than NGF + atRA as denoted by the letter l. But, NGF is not significantly different from atRA at E6.5-7 because there are not two of the same letters in these two groups.

thermore, mice with a targeted deletion of the BDNF gene show either no change or an increase in the number of neurons present in the sympathetic nervous system (51-53). The results show that BDNF alone did not support the survival of a significant number of neurons at any developmental stage. The addition of both atRA and BDNF slightly increased survival of E11 neurons when compared with BDNF alone, but the number of surviving neurons did not differ from the group treated with atRA alone. Therefore, atRA did not potentiate the effects of BDNF at any stage studied. This shows that atRA does not participate with neurotrophins indiscriminately, rather, it acts in concert specifically with NGF and NT-3, which are known to play a physiologic role in developing embryonic sympathetic neurons.

Increased Survival in the Presence of atRA Is Not Due to an Increase in Cell Proliferation. BrDU incorporation assays were conducted to determine whether or not the increase in cell number that occurred in the presence of atRA was due to longer survival of existing cells or to increased cell proliferation. As shown in Figure 2, the presence of atRA did not increase cell proliferation in the presence of either NGF or NT-3 at E6.5 or E11. Thus, atRA potentiates NGF- and NT-3-mediated survival by increasing the survival of existing cells. These results also demonstrate that the proliferation of chick sympathetic neuroblasts decreases with maturation *in ovo*, which agrees with observations in the mouse (32).

Neurite Outgrowth Is Enhanced by atRA in Em-

Table II. Statistical Analysis of Survival Differences* Due to Treatment Across Three Developmental Stages

| Stage | NGF | NGF + atRA | NT-3 | NT-3 + atRA |
|--------|-----|------------|------|-------------|
| E6.5-7 | a | e | f | g |
| E11 | a | b | c | d |
| E14 | a | b,e | c,f | d,g |

Note. Only vertically read comparisons can be made in this table. * Groups with the same letter differ significantly. For example, the amount of survival observed in the presence of NGF is significantly different at E6.5-7 when compared with E11 or E14 indicated by the fact that all three contain the letter a. However, survival in the presence of NT-3 is only different between E6.5-7 and E14 neurons as denoted by the letter f.

Embryonic Sympathetic Neurons Cultured in the Presence of NT-3 or NGF. The presence of atRA resulted in a dramatic enhancement of neurite outgrowth in cultures receiving either NT-3 or NGF (Fig. 3). However, the morphology of the neurites appeared somewhat different depending upon the neurotrophin that was used. Neurites appeared thicker and less branched in the NT-3 + atRA-treated cells when compared with the NGF + atRA-treated neurons. Whether there are intrinsic differences in the manner in which NT-3 and NGF influence process outgrowth in the presence of atRA remains to be established. However, it is clear that atRA enhances the extent of neurite outgrowth in the presence of NT-3 when compared with NT-3 alone.

Changes in Neurotrophin Receptor mRNA Levels. One possible mechanism whereby atRA could influence NT-3 or NGF signaling is by regulating the levels of neurotrophin receptor mRNAs. Therefore, studies were ini-

tiated to determine if the mRNAs encoding for *trkA*, *trkC*, or *p75^{NTR}* were altered in atRA-treated sympathetic neurons. Because of the limited amount of mRNA that can be collected from these cultures, it was necessary to use Q-PCR to assess the changes in steady-state levels of mRNA. Prior to initiating these experiments, the validity of the Q-PCR method was examined by measuring *trkA* mRNA in whole chick sympathetic ganglia at E6.5-7 and E11 and then comparing the results to those obtained using Northern analysis or RNase protection assay. Data obtained by Northern analysis was normalized to GAPDH and β -actin as described in Plum and Clagett-Dame (13), and that from the RNase protection assay was normalized to GAPDH, which corresponded to nucleotides 62 to 182 of the sequence described in Panabières *et al.* (39). The ratio of *trkA* mRNA at E11 to that at E6.5 was compared for each analytical technique. There was a 4-fold increase of *trkA* mRNA in ganglia taken from E6.5 versus E11 embryos as assessed by Q-PCR. The fold-induction observed by Q-PCR was in good agreement with the results obtained both by Northern analysis (4-fold increase from E6.5 to E11) and RNase protection assay (6-fold increase).

Following validation of the Q-PCR method, regulation of *trkA*, *trkC*, and *p75^{NTR}* mRNAs by atRA was studied in sympathetic neurons isolated from E6.5-7 embryos. A representative gel for each target mRNA studied by Q-PCR is shown in Figure 4. Figure 5 summarizes the Q-PCR results from three to seven independent culture experiments. The results show that treatment of neuronal cultures prepared from embryos at E6.5-7 for 72 hr with NGF + atRA resulted in a modest but reproducible increase in the steady-state

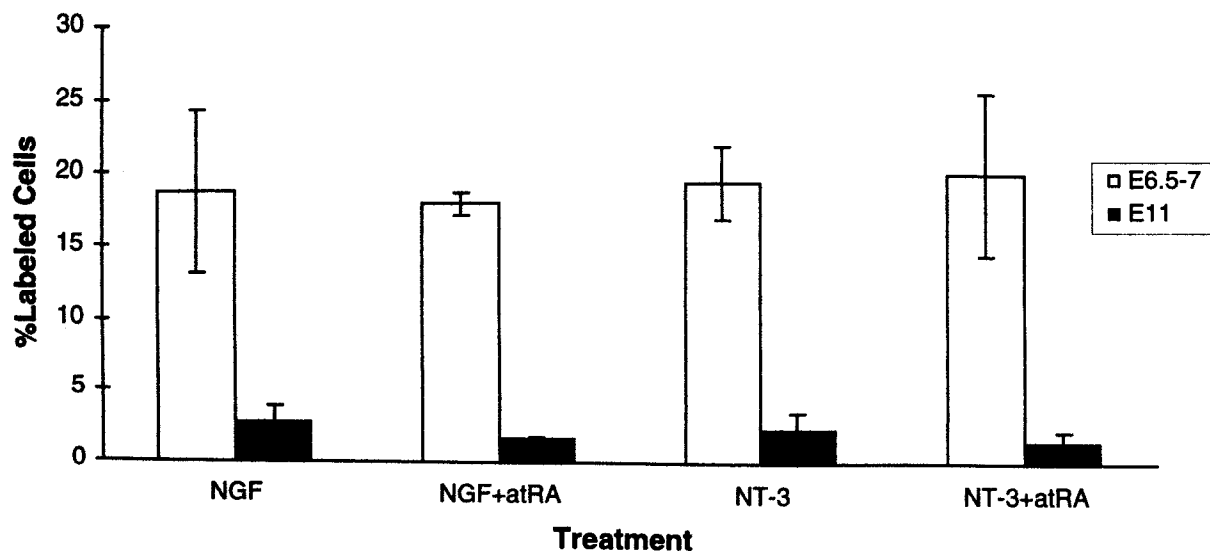


Figure 2. atRA does not stimulate cell proliferation in either NT-3- or NGF-treated sympathetic neurons. Primary cultures of sympathetic neurons (~25,000 cells/well) were prepared from chick embryos at two stages of development (E6.5-7 and E11). The cells were treated with 20 ng/ml neurotrophin alone or the combination of 20 ng/ml neurotrophin and 5×10^{-9} M atRA. Five hours later, BrDU was added and the cells were incubated another 12 hr before fixation. BrDU was detected immunocytochemically, and the number of labeled cells was determined in 12 random fields. Two to three independent culture experiments were done for each stage of development. Statistical analysis was performed using ANOVA followed by pairwise comparisons using Bonferroni and least significant difference *t* tests with significance set at $P < 0.05$. There were no statistical differences due to treatment within a developmental stage. However, the number of proliferating cells in all treatment groups at E11 was significantly lower than at E6.5-7. Values shown are mean \pm SE.

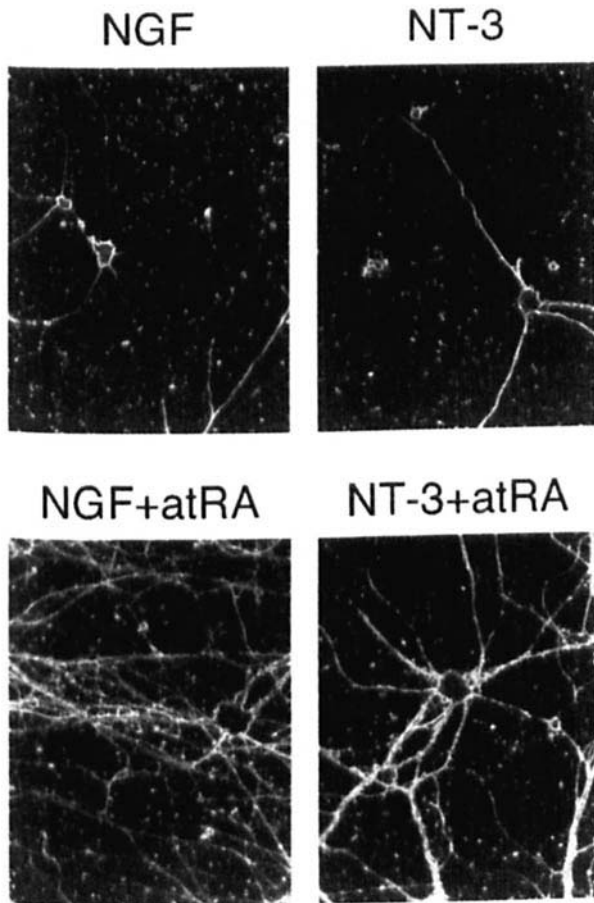


Figure 3. Neurite outgrowth is enhanced by atRA in NT-3-, as well as NGF-treated sympathetic neurons. E6.5-7 sympathetic neurons were treated with neurotrophin in the absence or presence of the indicated neurotrophin for 7 days, fixed, and stained with antibodies that detect neuronal cell bodies (ANNA) and neurites (neurofilament-M). Photomicroscopy was performed using darkfield illumination, and all photographs were taken at the same magnification (200 \times). The results shown here are representative of nine independent culture experiments.

levels of *trkA* mRNA (~ 1.5- to 2-fold). *TrkA* mRNA levels were not significantly increased above controls at earlier times (4, 12, 24, and 48 hr). The mRNA encoding for p75^{NTR} was significantly increased after exposure of cells to atRA for 48 hr. In contrast, *trkC* mRNA levels were not increased by atRA at any of the tested times.

It has been reported that NT-3 is required to induce *trkA* and NGF-responsiveness (4). For this reason, we also examined whether steady-state levels of *trkA* mRNA were altered in cultures exposed to NT-3. We did not observe any change in *trkA* mRNA after the exposure of E6.5-7 neurons to NT-3 alone for 24 hr (data not shown).

Because we were unable to detect any significant changes in neurotrophin receptor mRNAs until 48 to 72 hr after exposure to atRA, a gene that is known to respond rapidly to atRA (*RAR β 2*) was examined as a control to check for neuronal responsiveness at the earlier time points. As shown in Figure 5b, an increase in *RAR β 2* mRNA could be detected as early as 4 hr after atRA treatment. Thus, the inability to detect any change in the steady-state level of

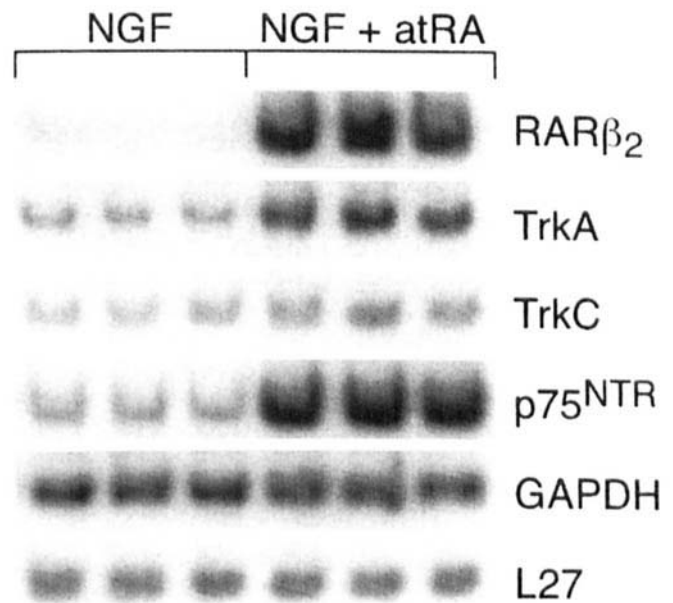


Figure 4. A representative gel for each of the target mRNAs studied by Q-PCR analysis in one batch of starting template. mRNA was isolated from neurons and was reverse transcribed as described in Materials and Methods. PCR reactions were prepared in triplicate for each treatment group and were analyzed for at least three cycles that were determined to fall within the linear range of amplification for each primer pair. The amount of starting template for each treatment set (NGF versus NGF + atRA) was equalized based on PCR analysis using GAPDH and/or L27 primers, which were determined to yield identical results. This figure shows the amount of ³²P-labeled PCR product obtained using template prepared from neurons treated for 72 hr with either NGF or NGF + atRA and analyzed after 26, 26, 26, 25, 24, or 22 cycles of PCR for *RAR β 2*, *trkA*, *trkC*, p75^{NTR}, GAPDH, and L27 respectively.

neurotrophin receptor mRNAs at the early time points was not due to a lack of atRA responsiveness in these cells or to suboptimal RNA quality of these samples. It is also interesting to note that *RAR β 2* mRNA was robustly induced in response to atRA in cultured sympathetic neurons regardless of whether neurotrophin was present or not (data not shown).

Discussion

The most striking observation from the present study is the increase in survival of embryonic chick lumbar sympathetic neurons that occurs when atRA is added to NT-3-containing cultures. As described previously, atRA also increases the NGF-dependent survival of these neurons, and the effect is particularly pronounced at early developmental stages. This suggests that during embryonic development, atRA may play a role in fine tuning embryonic responsiveness to both NT-3 and NGF.

The exact stage when the survival of developing sympathetic precursor cells and/or neurons is influenced by NT-3 is controversial, and there is evidence to support both early (4-6) and late (4, 8, 9) effects. In the present study, the survival of lumbar sympathetic neurons taken at either E6.5-7, 11, or 14 days and cultured for an additional 7 days was enhanced by the combination of either NT-3 and atRA or NGF

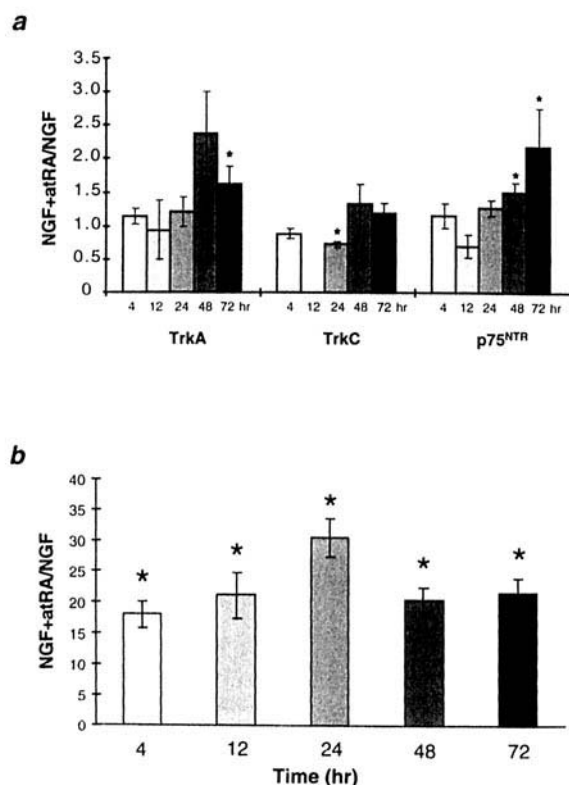


Figure 5. (a) atRA increases *trkA* and *p75^{NTR}* mRNA levels after 72 and 48 hr respectively, but does not increase *trkC* mRNA in E6.5-7 sympathetic neurons. (b) RARβ2 mRNA levels are increased within 4 hr after exposure to atRA. Neurons were treated with either NGF (20 ng/ml) or NGF + atRA (5×10^{-9} M), RNA was collected after various times of treatment and Q-PCR was performed as described in Materials and Methods. Changes in mRNA levels were analyzed using template produced from three to six independent culture experiments for each time point. Target gene analysis was performed on each template on at least two separate occasions and an average value was generated for each independent culture experiment. These values were used in the statistical analysis in which the bars with an asterisk were determined to be significantly ($P < 0.05$) different from a value of one (a value of 1 indicates no difference between mRNA level in the absence or presence of atRA, whereas a value different than 1 indicates fold change from the non-retinoid-treated sample).

and atRA when compared with neurotrophin or retinoid alone. This suggests that in the lumbosacral region of the chick, retinoid has the potential to modulate neurotrophin responses both early and late in the development of this cell type when placed in culture. The presence of RAR and RXR mRNAs at these developmental stages also supports a role for retinoids in the embryonic sympathetic ganglia of the chick (13). It will be important to determine the levels of retinoid to which developing sympathetic ganglia are normally exposed and how these levels change with development.

The mechanism whereby atRA enhances neuronal survival in the presence of NT-3 is unknown. The present work shows that atRA increases the number of neurons after 7 days in culture by enabling a greater number of existing cells to survive, as opposed to increasing proliferation of the initial cell population. This is true both at early stages of development when a high number of cells within the gan-

glion are proliferating, as well as at later stages when only a small percentage of cells are able to proliferate. In contrast, in the mouse, a proliferative response of sympathetic neuroblasts to atRA has been reported (E13.4 and E14.5), whereas later in development (E15.5), the number of proliferating cells decreases and the effect of atRA on proliferation becomes insignificant (32). However, the proliferative response of mouse neuroblasts only occurred at concentrations of 10^{-7} M atRA or greater, and was not observed at the level of atRA (5×10^{-9} M) studied in the present report.

The fact that atRA alone does not increase sympathetic neuronal survival indicates that atRA must interact in some way with the NT-3 signaling pathway. NT-3 is known to bind to and activate several *trk* family members, and it also interacts with *p75^{NTR}* (22). Although NT-3 binds to *trkC* with the highest specificity, the present work argues against regulation of *trkC* mRNA expression as a mechanism whereby atRA leads to increased neuronal survival in the presence of NT-3. This mRNA was not increased by exposure of neurons to atRA at any of the times studied (4, 24, 48, and 72 hr in culture). Our results agree with an earlier, somewhat limited, analysis showing a lack of effect of atRA on *trkC* mRNA levels in chick embryonic sympathetic neurons studied after 3 days in culture (21). We believe that an increase in *p75^{NTR}* mRNA is also unlikely to have contributed to enhanced neuronal survival in the presence of NT-3 + atRA, as elimination of this receptor has been shown to increase responsiveness to NT-3 in mouse sympathetic neurons (28).

NT-3 has also been shown to compete with NGF for binding to *trkA* (54–58) and numerous reports indicate that NT-3 can stimulate tyrosine phosphorylation of *trkA* and can support the survival and/or differentiation of cells lacking *trkC* (4, 7, 54, 58, 59–66). Thus, an increase in *trkA* mRNA represents one means whereby atRA could mediate enhanced neuronal survival in the presence of NT-3. Both our work and that of Holst *et al.* (21) support a retinoid-induced increase in *trkA* mRNA expression in chick sympathetic neurons, although the magnitude of the increase after 3 days of culture in our studies is much more modest than that reported previously (1.5-2 fold vs 6.9 fold, respectively). This increase in *trkA* mRNA is reported to account for the ability of atRA to increase NGF-mediated survival, and thus, may also play a role in neuronal response to NT-3. It is curious that work using cultured mouse embryonic sympathetic neuroblasts did not show a similar effect of atRA on *trkA* mRNA (32).

Our results show that *trkA* mRNA is not rapidly induced, as we find no significant change in this mRNA prior to 3 days of culture. In contrast, Holst *et al.* (21) report that *trkA* mRNA is increased in chick sympathetic neurons after exposure to atRA for only 3 hr. Thus, in our studies, the length of time required to see a change in *trkA* mRNA levels would suggest that atRA is not acting directly on this gene, rather, it may alter the transcription of intermediary gene

products. Additional studies to confirm that protein synthesis is required are not possible given the length of time (72 hr) required to see the mRNA increase. Treatment of neurons for this period of time with inhibitors of protein synthesis would result in neuronal death. A search for sequences with identity to known RAREs (67) in the human *trkA* promoter (68) also suggests that RARs are not directly involved in the regulation of this gene (Plum LA, Clagett-Dame M, unpublished data).

In the mammal, two *trkA* isoforms with and without an 18-bp insert in the extracellular domain have been found (69–73). When expressed in PC12^{nmr5} cells (a variant that lacks endogenous *trkA*), the longer form encodes for a receptor that is responsive to NT-3 (72). This is the only *trkA* isoform reportedly expressed in neuronal tissues in the rat and human (71). In the chick, a *trkA* isoform with some similarity to the mammalian form containing the six amino acid insert has been reported (73), however, the ability of this isoform to confer NT-3 binding or activation has not been tested. In the present work, atRA potentiated the activity of NT-3 used at a concentration of 2 ng/ml, a concentration that was shown to only marginally activate the NT-3-sensitive rat *trkA* isoform in PC12^{nmr5} cells. It is possible that this *trkA* isoform might be even more responsive to the effects of NT-3 in a normal neuronal context, or that the chick homologue might be more sensitive to NT-3 than its mammalian counterpart. Although an increase in *trkA* is proposed as one possible means whereby atRA could potentiate the survival-promoting effects of NT-3 in developing sympathetic neurons, we cannot rule out that other mechanisms may be involved, such as an effect on signaling components that lie downstream of the receptor mediating the NT-3 effect.

In conclusion, this work clearly shows that atRA in conjunction with NT-3 enhances both the survival and neurite outgrowth of sympathetic neurons explanted from the chick embryo at several developmental stages. Thus, retinoids have the ability to modulate the responsiveness to both NGF and NT-3 in the developing sympathetic nervous system of the embryonic chick.

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