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# NEUROTROPHIC FACTOR-LIKE ACTIVITY IN *DROSOPHILA*

Izumi Hayashi, Martha Perez-Magallanes and John M. Rossi

Department of Molecular Genetics, Beckman Research Institute of the City of Hope
Duarte, California 91010

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**Summary:** The NGF-family of neurotrophic factors are structurally similar peptides with related functional properties. So far, this family of neurotrophic factors has only been identified in the vertebrate nervous system. We have determined that cultured *Drosophila* embryonic cells produce and secrete into medium, an activity which stimulates neurite outgrowth of embryonic chick sensory ganglia. This *Drosophila* activity can be blocked by antibodies to mouse NGF, indicating an immunological relationship between the *Drosophila* factor, mouse NGF and possibly other vertebrate neurotrophic factors. Addition of mouse NGF to *Drosophila* embryonic cells in culture results in increased cell number and enrichment of the neuronal phenotype, indicating that *Drosophila* cells have the ability to respond to the vertebrate factor. In addition, poly(A)$^+$RNA extracted from *Drosophila* contains a single 1.4 Kb band which cross-hybridizes with a mouse NGF cRNA probe. These results indicate that vertebrate neurotrophic factor-like functions may operate in a genetically defined invertebrate species. 

The NGF-family of neurotrophic factors regulate differentiation of the vertebrate nervous system and maintenance of its various functional properties. Nerve growth factor (NGF), the first neurotrophic factor to be discovered, was originally described as an agent which stimulated the neurite outgrowth of chick embryonic sympathetic and sensory ganglia (1). A large body of studies since then has established that NGF is essential for the survival and maintenance of specific populations of differentiated neurons derived from the neural crest (2). NGF has also been shown to have trophic interactions on central cholinergic neurons (3), widening its range of targets on differentiated neuronal cell populations.

A less appreciated function of NGF is its ability to stimulate cell division. A mitogenic role for NGF acting on embryonic, mitotically active neuron precursor cells, was described in the early days of NGF history (4). This function has found strong support in recent years by the demonstration of a mitogenic effect of NGF on neural crest-derived cell lines (5), neuro-epithelial stem cells (6) and pluripotent adrenal chromaffin cells (7).

The specificity of NGF action on subsets of cells in the nervous system suggested the existence of other neurotrophic factors, which led to the discoveries of brain-derived neurotrophic factor (BDNF, ref.8) and neurotrophin-3 (NT-3, ref.9). All three neurotrophic factors possess neurite outgrowth promoting activity on embryonic chick dorsal root ganglion explants (10). Biological activities so far examined suggest distinct as well as overlapping properties for each neurotrophic factor (11).

All three neurotrophic factors sharestructural similarities. Comparison of NGF's among different species (12) also indicate that neurotrophic factors are evolutionarily conserved. The significance of

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these similarities for understanding the primary role of neurotrophic factors in nervous system development is yet to be elucidated.

Existence of a molecule(s) functionally or biochemically related to vertebrate neurotrophic factors has not been reported in invertebrates. Here we report that *Drosophila* possesses neurotrophic factor-like functional activity. Biochemical and molecular characterization of this *Drosophila* activity will ultimately allow us to dissect the fundamental role of neurotrophic factors in nervous system development in an organism amenable to genetic analysis.

## Materials and Methods

***Drosophila embryonic cell culture.*** *Drosophila* Canton S embryonic cells were prepared for culture by Seecof's method (13). Briefly, the embryos were collected for 2 hours on standard corn meal-agar plates and allowed to develop for 3 hours at 25°C. The early gastrula stage embryos were dechorionated in 1.5% sodium hypochlorite/47.5% ethanol, and homogenized with a Dounce homogenizer in Schneider's modified *Drosophila* medium, supplemented with 18% fetal calf serum and 0.2μg/ml bovine insulin. The homogenate was passed through a Nytex mesh filter (50 μ) and the single cells were plated in the above medium. After attachment (15 minutes after plating), the medium was changed to experimental conditions. The cultures were maintained at 25°C.

***Analyses of NGF effects in Drosophila embryonic cells in culture.*** Cell growth was assessed by counting cells 8 hours after plating, when the majority of the cells have ceased to divide but have not yet differentiated. Cultures were trypsinized, stained with trypan blue and cells were counted with a hemocytometer. To assess differentiation, myotubes and neuron clusters were scored from terminally differentiated cultures 24 hours after plating. Cells were fixed in 4% paraformaldehyde and counted through a calibrated reticle in the ocular lens of a phase contrast microscope. Ten fields were examined for each samples. Mouse NGF (2.5S NGF, culture grade) was obtained from Collaborative Research. Affinity purified sheep anti-mouse NGF antiserum (14) was used at 250 ng/ml.

***Assay for neurite outgrowth promoting activity.*** Dorsal root sensory ganglia were isolated from 8 to 10 day old chick embryos and placed on Falcon Premaria dishes in the presence of small quantities of fresh culture medium (1:1 mixture of serum-free RPMI 1640, and Schneider's modified *Drosophila* medium supplemented with 5% fetal calf serum). After attachment of the ganglia (about 30 minutes), 0.5 ml of experimental medium was added to each dish. The ganglia were cultured at 37°C in a humidified $CO_2$ incubator for 24 hours. The results were photographed using an Olympus Vanox phase contrast microscope, after fixation of the ganglia in 4% paraformaldehyde. *Drosophila* culture conditioned medium was obtained as follows: Gastrula stage cells were cultured as described above, except that the cells were plated at 4 x$10^6$ cells per 60 mm dish in fresh Schneider's modified *Drosophila* medium supplemented with 5% fetal calf serum. After 5 days in culture, the medium was collected, centrifuged, passed through a 0.2 μ Gelman filter and used immediately.

***Northern analysis of Drosophila poly(A)$^+$RNA.*** *Drosophila* poly(A)$^+$RNA samples were analyzed using a $^{32}$P-labelled cRNA probe derived from a mouse NGF cDNA clone, 12E4 (15). Poly(A)$^+$RNA was glyoxalated and electrophoresed overnight through a 1.5% agarose gel and transferred onto a nitrocellulose membrane. Hybridization was performed overnight at 60°C in 50% formamide, 5XSSC, 50 mM NaPi, pH6.5, 0.5X Denhardt's, 0.1%SDS, 1mM EDTA, 250 μg/ml salmon sperm DNA, and 10 μg/ml *Drosophila* 3rd instar poly(A)$^-$RNA. The NGF probe was used at $10^6$ cpm/ml of hybridization buffer. Washing was in 2XSSC, 0.1% SDS at room temp. for 30 minutes twice, followed by a final wash at 60°C for 20 minutes in 0.1X SSC, 0.1% SDS. *Drosophila* ß-tubulin cRNA probe was used to quantitate poly(A)$^+$RNA samples from different stages of development, since ß-tubulin mRNA is found at constant levels throughout *Drosophila* development (16). After stripping the mouse NGF cRNA probe hybridization in 0.1XSSC, 0.1% SDS at 80°C for 30 min, the tubulin probe was used as described above. The final wash was at 65°C for 30 minutes in 0.1X SSC and 0.1% SDS.

## Results and Discussion

***Drosophila embryonic cells in culture produce neurite outgrowth promoting activity.*** The classic *in vitro* neurite extension assay of chick embryonic sensory ganglia (1) was used to examine whether *Drosophila* cells produce a neurite outgrowth promoting activity similar to vertebrate neurotrophic factors. When dorsal root sensory ganglia from chick embryos were grown in fresh culture medium, no neurite outgrowth was observed as shown in Figure 1A. Addition of mouse NGF to this medium resulted in a characteristic neurite halo (Figure 1B). The mouse NGF-induced neurite outgrowth was inhibited by concomitant addition of anti-mouse NGF antibodies (Figure 1C).

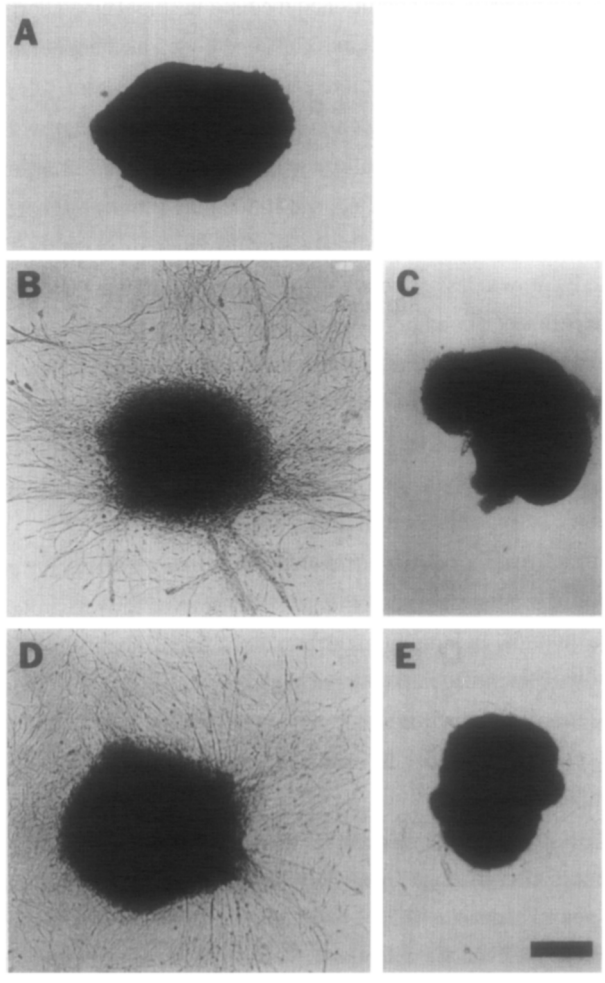


**Figure 1.** Neurite outgrowth promoting activity of conditioned medium from *Drosophila* embryonic cells in culture on chick embryonic dorsal root ganglion explants. **A:** Fresh culture medium (1:1 mixture of Schneider's modified *Drosophila* medium supplemented with 5% fetal calf serum and serum-free RPMI 1640); **B:** Fresh culture medium + mouse NGF; **C:** Fresh culture medium + mouse NGF + anti-mouse NGF antibodies; **D:** 1:1 mixture of serum-free RPMI 1640 and *Drosophila* culture conditioned medium; **E:** Medium as in D, with anti-mouse NGF antibodies. Mouse NGF: 20 ng/ml; anti-mouse NGF antibodies: 250 ng/ml. The bar at the right bottom indicates 0.2 mm.

Addition of *Drosophila* conditioned medium, obtained from 5-day cultures of *Drosophila* gastrula stage embryonic cells, also resulted in stimulation of significant neurite outgrowth in the absence of any mouse NGF (Figure 1D). The neurite outgrowth in the presence of *Drosophila* conditioned medium was blocked by anti-mouse NGF antibodies (Figure 1E). These results indicate that *Drosophila* cells in culture produce a factor which is functionally similar to vertebrate neurotrophic factors in stimulating neurite outgrowth of chick embryonic sensory ganglia. In addition, the results suggest that the factor produced by *Drosophila* cells shares immunological homology to mouse NGF, since the neurite outgrowth promoting effect of the conditioned medium was blocked in the presence of antibodies to mouse NGF. Neurite outgrowth observed in the presence of *Drosophila* conditioned medium was not due to increased spreading of the connective tissue cells, since no neurite outgrowth was observed in the presence of anti-mouse NGF in spite of a similar spreading of connective tissue cells in such cultures. The effect of the conditioned medium in stimulating neurite outgrowth increased with the age of the conditioned medium. The conditioned medium obtained from 5 day-old cultures of *Drosophila* embryonic cells was more effective than those from 3-day or 1-day cultures (data not shown). This could be due to the accumulation of the neurite outgrowth promoting activity in the conditioned medium over prolonged culture period for *Drosophila* embryonic cells, or it could be due to an increase in the production of the neurite stimulating activity by more differentiated and older cells in the culture. It should be noted that *Drosophila* conditioned medium, although qualitatively similar to mouse NGF, was less effective than the purified growth factor in stimulating neurite outgrowth under the experimental conditions employed. This may be due in part to the conditions for obtaining *Drosophila* conditioned medium or alternatively, the less-than-optimal effect of the *Drosophila* conditioned medium could be due to the biochemical nature of the *Drosophila* factor. Examination of these different possibilities is technically not feasible without a partial purification of the *Drosophila* neurite outgrowth promoting activity, due to the differences in culture conditions between the *Drosophila* embryonic cells and embryonic chick ganglia.

***Mouse NGF stimulates division of Drosophila embryonic neuron precursor cells in culture.*** Highly reproducible primary cultures can be obtained from cells of early gastrula stage *Drosophila* embryos (13). In these cultures, an initially undifferentiated population of cells undergoes rapid multiplication and terminally differentiate into multinucleated myotubes and differentiated neurons. The *Drosophila* culture system offers a unique opportunity to examine the effects of various epigenetic substances on the early phase of neuronal development, when precursor cells of the nervous system are still mitotically competent (17, 18).

Addition of mouse NGF to the culture medium resulted in a 2.4 fold increase in cell number over the control cultures 8 hours after plating (Figure 2A). Addition of mouse NGF did not affect the percentage of trypan blue positive, dead cells at 8 hours (about 5% in all cultures), indicating that the stimulatory effect of mouse NGF on the cell number is more likely to be due to an increase in cell division rather than an increase in the number of viable cells. The mouse NGF effect was concentration dependent, and was most effective at 2 ng/ml ($10^{-10}$M). Concomitant addition of mouse NGF and anti-mouse NGF antibodies abolished the NGF effect on the cell number increase (Figure 2B). These results indicate that the increase in cell number observed after NGF addition to *Drosophila* embryonic cell cultures is due to NGF itself, and not to possible contaminants in the NGF prepara-

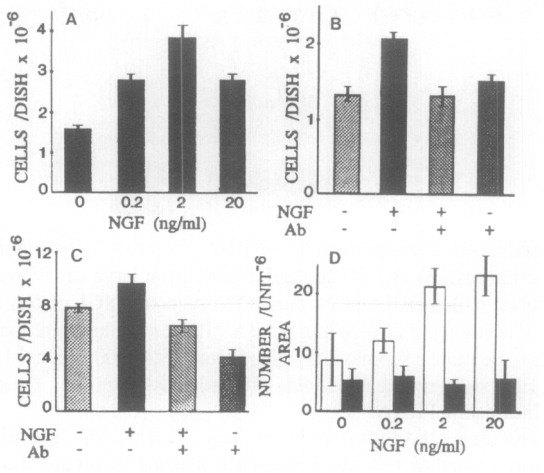


**Figure 2.** Effect of mouse NGF and anti-mouse NGF antibodies on the growth and differentiation of *Drosophila* gastrula stage embryonic cells in culture. **A:** Effect of mouse NGF on cell proliferation. Cells were plated at 0.8 X $10^6$ per 35 mm culture dish. **B:** Supression of growth-stimulating effect of mouse NGF by anti-mouse NGF antibodies. Cells were plated at 0.5 X $10^6$ per 35 mm culture dish. **C:** Supression of cell growth by anti-mouse NGF antibodies in high density cultures. The cells were plated at 2 X $10^6$ per 35 mm culture dish. Note that the antibodies inhibited growth not only in the presence, but also in the absence of mouse NGF. **D:** Effect of mouse NGF on the differentiation of *Drosophila* embryonic cells. An average of 10 fields per plate were examined and scored for neuron clusters and myotubes, each with randomly chosen different unit area of 500 $\mu m^2$. This experiment was done in parallel with the experiment in **A**. For all experiments, bars represent the mean of counts from duplicate plates with standard error.

tion. The results also suggest that the *Drosophila* cells may possess a receptor which can recognize mouse NGF. Anti-mouse NGF antibodies alone did not have any effect on cell number in these cultures. However, when the cells were cultured at high densities, the anti-mouse NGF antibodies not only suppressed the increase in cell number observed in the presence of mouse NGF, but also inhibited the cell number increase by 50% in the control cultures to which no NGF had been added(Figure 2C). The effect of mouse NGF on cell growth was also less evident in high density cultures (Figure 2C). These results, in addition to the neurite outgrowth promoting activity of the *Drosophila* cell culture conditioned medium, suggest the presence of a neurotrophic factor in the *Drosophila* culture medium. *Drosophila* cells in culture appear to be producing and responding to their own trophic factor whose effect can be mimicked or replaced by mouse NGF. Anti-mouse NGF antibodies appear to interfere with the action of the *Drosophila* trophic factor present in the high density cultures.

The increase in cell number observed in the presence of mouse NGF seems to result from the preferential stimulation of the growth of neuronal precursor cells. Cells that form terminally differentiated neuron clusters are clonally related, arising from a single neuron precursor cell (17). Hence, our results which show an increase in the number of neuron clusters per unit area in the presence of mouse NGF (Figure 2D), strongly suggests an underlying increase in the number of neuron precursor cells. The number of myotubes per unit area remained constant.

Several mechanisms are possible which could result in the increase in cell number and the subsequent enrichment of the neuronal phenotype of the *Drosophila* cultures in the presence of mouse NGF. Mouse NGF (or the equivalent *Drosophila* factor) may stimulate the rate of cell division or prolong the duration of the growth phase of *Drosophila* neuron precursor cells. Alternatively, the

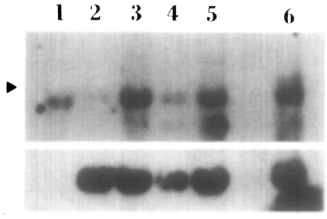


**Figure 3.** Northern analysis of *Drosophila* poly(A)$^+$RNA. Top panel: *Drosophila* poly(A)$^+$RNA samples from (2) 5hr embryos, (3) 1st, (4) 2nd and (5) 3rd instar larva and (6) young adults (within 1 week of hatching), probed with a cRNA probe derived from mouse NGF cDNA clone, 12E4. Numbers represent lanes. Twenty µg of *Drosophila* poly(A)$^+$RNA samples were loaded in each lane. Lane (1) is male mouse submaxillary gland total RNA (300 ng), used as a standard. Bottom panel: The same filter probed with *Drosophila* ß-tubulin cRNA probe. The arrow indicates the position of 16S *E. coli* ribosomal RNA.
The mouse NGF cDNA clone 12E4 (gift from Dr. A.Gray, Genentech), originally carried in a pBR322 plasmid vector, was subcloned into a Bluescribe plasmid vector and the cRNA probe was synthesized from the *EcoRI*-linearized Bluescribe clone using T3 RNA polymerase in the presence of $^{32}$P-UTP (New England Nuclear, 3000 Ci/mol). For the tubulin probe, a 398 bp *DraII-EcoRV* subfragment of the ß-tubulin cDNA pTu56 (gift of Dr. R. Renkawitz-Pohl, Max-Planck Institute, Munich) was subcloned into Bluescript plasmid vector and the cRNA probe was synthesized from the *EcoRV*-linearized clone.

factor may play a role in influencing the *Drosophila* gastrula stage embryonic cells to take a neuronal pathway, thus increasing the proportion of cells which become neuron precursor cells, as was recently suggested with BDNF for pluripotent neural crest-derived cells in the chick embryo (19). These different possibilities will have to be examined further by detailed analyses of neurotrophic factor effect(s) on the growth and differentiation properties of the *Drosophila* embryonic cells in culture.

***A Drosophila mRNA is recognized by a mouse NGF probe.*** In order to obtain additional molecular evidence for a *Drosophila* factor that resemble vertebrate neurotrophic factors, *Drosophila* poly(A)$^+$RNA was examined by Northern blot analysis, using a probe specific for mouse NGF mRNA. The mouse probe hybridized to a single band of *Drosophila* poly(A)$^+$RNA in all stages of development examined (Figure 3). Cross hybridization of a *Drosophila* mRNA band with the mouse NGF probe under stringent cross-species hybridization conditions suggests a strong similarity in the sequence of mouse NGF and the *Drosophila* mRNA band. The size of the *Drosophila* mRNA (1.4 Kb) was also similar to that of the mouse ßNGF mRNA (1.3 Kb), as well as to other known vertebrate neurotrophic factor mRNAs (8,9). In addition, the level of the *Drosophila* mRNA appeared to change as a function of developmental stages. Significantly, the mRNA was already detectable in 5 hour embryos, when there is active multiplication of neuron precursor cells but no overt detectable neuronal differentiation. The *Drosophila* mRNA increased dramatically in the larval stages, coincident with our observations on the neurite outgrowth promoting activity of the 5 day-old conditioned medium (*in vivo* equivalent of the late larval stage cells).

***Concluding Remarks.*** Our studies demonstrate the existence of a *Drosophila* molecule with both structural and functional similarities to the vertebrate neurotrophic factors. Many factors in the past have been shown to stimulate neurite outgrowth of chick embryonic sensory ganglia, and in this regard, the neurite outgrowth promoting activity of the *Drosophila* conditioned medium itself is not surprising. However, in our studies, actions of this factor on *Drosophila* cells can be mimicked by

mouse NGF, the most extensively studied and readily available neurotrophic factor of the NGF-family of peptides.

Much of our present knowledge on the actions and localizations of vertebrate neurotrophic factors are based on NGF. Recent studies on the NGF-family of neurotrophic factors suggest, that many of the earlier studies attributing the functional activity to NGF may require reevaluation. These include indications that NGF, BDNF and NT-3 may use the same receptors to mediate their effects (20, 21), and the possible cross reactivity of anti NGF antibodies to NT-3 and BDNF (22). In our present studies, we have used mouse NGF as a point of reference for the *Drosophila* neurotrophic activity. This does not preclude possibilities that the *Drosophila* factor may turn out to be more similar to the other members of the vertebrate neurotrophic factors, or found to be quite unique with limited but significant similarity to all vertebrate neurotrophic factors. The determination of the nature of the *Drosophila* molecule, and the extent of its homology to vertebrate neurotrophic factors must come from sequencing studies. Information gained from such studies may also bring insight into understanding the structure-function relationship and the evolutionary aspect of the vertebrate neurotrophic factors.

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