Growth cone responses to growth and chemotropic factors

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Abstract
During nervous system development axons reach their target areas under the influence of numerous guidance cues that affect rate and direction of growth. This report addresses the unsettled question of whether and to what extent growth velocity and turning responses (attraction, repulsion) are interdependent. We exposed individual growth cones of fetal rat dorsal root ganglion neurons in culture asymmetrically to gradients of seven different factors and recorded their growth rates and turning angles. Growth cones exhibited divergent patterns of turning and growth responses. For example, hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1) and thrombin all promoted growth, but HGF was a powerful attractant, thrombin a potent repellent and IGF-1 did not elicit turning. Galanin and neuropeptide Y also affected growth and/or turning differentially. Finally, nerve growth factor in the culture medium not only inhibited the turning responses to HGF, but also converted growth promotion of HGF and IGF-1 into inhibition. Overall, our studies indicate that: (i) turning and advance are regulated independently, except that strong attractive or repulsive responses generally are accompanied by growth promotion; (ii) asymmetric growth factor application per se does not elicit attraction; (iii) regulation of the two parameters may occur through a single receptor; and (iv) the effects of combined growth factors may not be additive and can be inhibitory.

Introduction
Nerve growth cones respond to a variety of microenvironmental cues that control their advancement and growth direction (see, e.g. Tessier-Lavigne & Goodman, 1996). These cues may be soluble or substrate-bound and may elicit simple growth responses (increased or decreased growth rate), chemotactic responses (turning toward source of factor/positive chemotropism) or chemorepellent responses (turning away from source of factor/negative chemotropism). However, neuron and growth cone reactions to a single factor can be complex. For example, nerve growth factor (NGF) can operate not only as a differentiation and growth factor, but also as an attractant (Kater et al., 1988; Henley & Poo, 2004). This has been shown for other factors since [e.g. brain-derived neurotrophic factor (BDNF) and netrin-1; Stein & Tessier-Lavigne, 2001; ]. Yet, other communications reported that growth rate and direction might not be linked. For example, in Xenopus spinal neurons BDNF and acetylcholine (ACh) trigger an attractive response without enhancing outgrowth (Zheng et al., 1994; Song et al., 1997), and Slit promotes outgrowth without eliciting turning (Stein & Tessier-Lavigne, 2001). This raises the question of the degree to which growth and turning responses are interdependent. Other studies revealed cross-talk between different cues, such as the inhibition of semaphorin 3A (Sema3A)-mediated chemorepulsion by NGF (Tuttle & O’Leary, 1998; Dontchev & Letourneau, 2002; Chalasani et al., 2003) or the inhibition of netrin-induced turning by Slit (Stein & Tessier-Lavigne, 2001). Such observations add to the complexity of the issue.

The data just reviewed come from a variety of experimental systems studied with diverse approaches. In combination, they generate an incoherent picture. In other words, the question of the degree to which attraction and growth promotion, on the one hand, and repulsion and growth inhibition, on the other hand, are correlated has not been investigated systematically, especially not in mammalian neurons. Therefore, we decided to address this issue by exposing individual, identified mammalian growth cones, in a chemically defined medium and under identical conditions, to gradients of different factors using the approach developed by Poo and collaborators (Lohof et al., 1992). These assays generated, over relatively short observation times, quantitative data for growth cone turning angles as well as for the rate of growth in response to specific factors. Using NGF-responsive neurons of rat dorsal root ganglia (DRG), we compared the reactions of growth cones to seven different factors that can be grouped as follows. (i) Classic growth factors: the neurotrophin, NGF; a broadly acting growth factor, IGF-1 (insulin-like growth factor-1); and the growth and chemotactic factor, HGF (hepatocyte growth factor). (ii) Neuropeptides: galanin and NPY (neuropeptide Y). (iii) Repellents: Sema3A and thrombin, which is known primarily as a platelet- and fibroblast-activating protease factor (Fenton, 1988). These compounds are highly diverse and have been reported to influence axonal growth (for review, see Table 1). By quantifying growth cone turning and advancement under identical conditions we were able to assemble a sizeable catalog that describes a single mammalian growth cone’s response patterns to various factors. This allowed us to begin to answer the question of the degree to which changes in growth rates are linked to turning responses. We also show effects of combining...
TABLE 1. Effects of factors on neurite growth

<table>
<thead>
<tr>
<th>Factor</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGF</td>
<td>Growth promotion</td>
<td>Cohen et al. (1954); Thoenen &amp; Barde (1980)</td>
</tr>
<tr>
<td></td>
<td>Chemoatraction</td>
<td>Letourneau (1978); Gunderson &amp; Barrett (1980)</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Growth promotion, membrane expansion at growth cone</td>
<td>Caroni &amp; Grandes (1990); Pfenniger et al. (2003)</td>
</tr>
<tr>
<td>HGF</td>
<td>Growth promotion</td>
<td>Maina et al. (1997); Korhonen et al. (2000)</td>
</tr>
<tr>
<td>Galanin</td>
<td>Growth promotion</td>
<td>Mahoney et al. (2003); Hobson et al. (2006)</td>
</tr>
<tr>
<td>NPY</td>
<td>Growth promotion</td>
<td>White (1998); Hansel et al. (2001)</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Growth inhibition</td>
<td>Hawkins &amp; Seeds (1986); Suidan et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>Chemorepulsion</td>
<td>de La Houssaye et al. (1999)</td>
</tr>
</tbody>
</table>

HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor-1; NGF, nerve growth factor; NPY, neuropeptide Y; Sema3A, semaphorin 3A.

different growth factors and approach the question of whether multiple receptors are needed for the growth and the turning responses.

Materials and methods

Materials

Reagents and their sources were: culture media, media supplements and laminin (Invitrogen, Carlsbad, CA, USA). Sema3A: the culture supernatant of stably transfected HEK 293 cells secreting Sema3A (generous gift from Dr M. Tessier-Lavigne) was concentrated by ultrafiltration (Centriplus membrane, 50 000 MW cut-off; Millipore, Bedford, MA, USA) and concentration calibrated by bioassay (Gatlin et al., 2006). Galanin (Bachem, Bubendorf, Switzerland); NPY (Bachem, King of Prussia, PA, USA); H 409/22 (AstraZeneca

Through Dr M. Nordlander, Gothenburg, Sweden); HGF (EMD Biosciences/Calbiochem; San Diego, CA, USA); IGF-1 and thrombin (Sigma, St Louis, MO, USA); NGF (Alomone Labs, Jerusalem, Israel).

DRG neuron culture

This study was approved by the Animal Care and Use Committee, UCHSC. Time pregnant females were deeply anesthetized with Halothane or Isoflurane. After removal of the uterus by caesarean section, the rats were killed while under anesthesia by cutting the heart. The removed fetus was decapitated prior to dissection. For explant cultures, DRG were dissected from 15-day gestation Sprague-Dawley rat fetus and cultured on laminin-coated coverslips (Assistent brand) in Neurobasal medium supplemented with B27, 10% v/v fetal bovine serum and 100 ng/mL NGF (3.8 nM). After 24 h incubation (37°C, 5% CO2 in air) this medium was replaced with fresh Neurobasal medium + B27, without other supplementation. After a second day in culture, neurites with spread growth cones were used for assays.

Growth and turning assays

Gradients of the factors were generated in the proximity of cultured nerve growth cones by repetitive-pulse application (Lohof et al., 1992). Micropipettes (inner diameter of the tip consistently 1–2 μm) were connected to a Picospritzer (set at 6 p.s.i.; General Valve, Fairfield, NJ, USA) controlled by a square wave generator (2 Hz, duration 10 ms; Astro-Med, West Warwick, RI, USA). The system was calibrated by generating a model gradient of fluorescein-conjugated dextran. By tightly controlling factor concentration in the micropipette, frequency of ejections, volume of ejected factor solution and position of the pipette tip relative to the growing neurite (see below), we generated such gradients highly reproducibly, and they were stable over time. Culture coverslips were placed in an Attocell cell chamber (Molecular Probes/Invitrogen, Carlsbad, CA, USA) with medium, and layered-over with inert mineral oil (embryo-tested, sterile-filtered; Sigma) to maintain pH and avoid evaporation.

Media and factor concentrations used are listed in Table 2. The effective concentrations of the factors at the growth cone were two- to sixfold over the reported receptor KD. Under most circumstances, the culture medium present during the assays consisted of serum-free Neurobasal medium supplemented with B27, a mixture consisting

TABLE 2. Summary of experimental conditions

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Culture medium</th>
<th>Factor gradient*</th>
<th>Receptor KD</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>DRG culture medium (Neurobasal/B27)</td>
<td>Thrombin (1 μM)</td>
<td>0.26 nM</td>
<td>Granick et al. (1994)</td>
</tr>
<tr>
<td>pCEP4 (control for Sema3A)</td>
<td>Neurobasal/B27</td>
<td>HGF (560 nM)</td>
<td>0.35 nM</td>
<td>Bussolino et al. (1992)</td>
</tr>
<tr>
<td>Sema3A</td>
<td>Neurobasal/B27</td>
<td>IGF-1 (5 μM)</td>
<td>0.89 nM</td>
<td>Freund et al. (1993)</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Neurobasal/B27</td>
<td>NPY (1 μM)</td>
<td>0.15–0.45 nM</td>
<td>Daniels et al. (1995)</td>
</tr>
<tr>
<td>HGF</td>
<td>Neurobasal/B27</td>
<td>Galanin (1 μM)</td>
<td>0.2 nM</td>
<td>Skofitsch et al. (1986)</td>
</tr>
<tr>
<td>NPY</td>
<td>Neurobasal/B27</td>
<td>NGF (1.9 μM)</td>
<td>0.2–0.3 nM</td>
<td>Banerjee et al. (1973); Herrup &amp; Shooter (1973)</td>
</tr>
<tr>
<td>NGF + HGF</td>
<td>Neurobasal/B27 w/3.8 nM NGF</td>
<td>HGF (560 nM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NGF + IGF-1</td>
<td>Neurobasal/B27 w/10 nM insulin</td>
<td>IGF-1 (5 μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H 409 + NPY</td>
<td>Neurobasal/B27 w/100 nM H 409</td>
<td>NPY (1 μM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Factor concentrations indicated for the micropipette (note that corresponding concentrations at the growth cone are approximately 1000 × lower). Concentration calibrated by bioassay (Gatlin et al., 2006). DRG, dorsal root ganglia; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor-1; NGF, nerve growth factor; NPY, neuropeptide Y; Sema3A, semaphorin 3A.
primarily of antioxidants, hormones (corticosterone, progesterone, insulin), vitamins and essential fatty acids (Brewer et al., 1993). To ascertain that B27 did not interfere with our growth cone experiments, we measured rates of advancement in the presence or absence of B27 in the culture medium. There was no detectable difference between the two conditions (data not shown), indicating that B27 did not promote or inhibit growth. Because high levels of insulin may activate the IGF-1 receptor, we generated a low-insulin environment for the assays with IGF-1 by using instead of B27 a low-insulin (10 nm) modification of N2 supplement (Bottenstein & Sato, 1979). In assays involving combinations of a factor with NGF, the neurons were grown in Neurobasal medium supplemented with B27 and 100 ng/mL (3.8 nM) NGF.

Neurons were observed on the microscope stage under convective heating at a constant 37°C. At the start of each experiment, the tip of the loaded micropipette was positioned 100 μm away from the selected growth cone, at an angle 45° from the initial direction of growth cone advance (as determined by the orientation of the growth cone’s neurite shaft). Initiation of factor expulsion marked the start (time t = 0) for each experiment. Phase contrast images were captured at 5-min intervals over the course of 1 h. To be scored, growth cones had to advance a minimal distance of at least 10 μm. Once this criterion had been met, growth cones were tracked for 1 h or until they either stopped (i.e. no advancement for ≥10 min) or branched. Growth rates were calculated as net distance traveled by individual growth cones over the first 30 min as well as the total observation.

![Image](https://example.com/image1.png)

**Fig. 1.** (A) Examples of growth cone responses to gradients of control medium and various factors (phase contrast micrographs). From left to right: initial position of a growth cone (+) relative to micropipette tip (*); position of a growth cone after 60 min in a control gradient; in a hepatocyte growth factor (HGF) gradient (60 min); in a thrombin gradient (60 min). White line indicates initial axis of growth. Black line connects growth cone end point with initial position and defines h, the final turning angle. Fetal rat DRG neurons grown on laminin. Scale bar: 20 μm. (B) Rosebud plots of axonal responses. Plots illustrate growth cone translocation during gradient exposure for 1 h. Arrows mark the position of the micropipette tip. Abscissa indicates distance in μm, and the ordinate marks 0° in an arc from −90° to +90°. (C) Cumulative distribution plots of turning angles in response to (from left to right) thrombin, control, insulin-like growth factor-1 (IGF-1) and HGF gradients.
time. Turning responses are shown as rosebud plots (the origin of the plot, 0 on the abscissa, represents the initial position of the growth cone) or as final turning angles. The final turning angle of the growth cone was the angle formed between the initial axis of outgrowth and a line drawn from the initial to the final position of the growth cone (Fig. 1A). Because all data sets exhibited normal distributions, and results obtained with a specific factor were compared with their controls, we assessed for statistical significance of final turning angles and growth rates with Student’s t-test (assuming equal variances).

Microscopy

All images were captured using a Zeiss Axiovert 200M microscope equipped with Zeiss optics, a Cooke Sensicam digital camera, and either Slidebook (Intelligent Imaging Innovations, Denver, CO, USA) or METAMORPH software (Molecular Devices, Sunnyvale, CA, USA).

Results

In order to assess the effect of a particular factor on growth cone turning, microgradients were generated as explained. Figure 1A shows the position of the micropipette tip relative to the growth cone, at the beginning of the experiment and after 60 min in control, attractant and repellent gradients. The gradients generated for these studies were consistent from experiment to experiment (see also Lohof et al., 1992). Tracking the positions of individual growth cones over time allowed us to assess quantitatively both their turning angles and rates of advancement. Turning results are shown as rosebud plots (Fig. 1B) and as the average final turning angles (initial direction of growth, white line; final growth direction, black line; Fig. 1A). The rosebud plots map the growth cones’ movements throughout the course of the experiment.

Growth cone turning angles

Growth cones may exhibit four different responses when exposed to the concentration gradient of a factor: (i) attraction – the growth cone turns towards the micropipette tip; (ii) repulsion – the growth cone turns away from the micropipette tip; (iii) indifference – the growth cone continues to grow more or less in its initial path; or (iv) the growth cone stops advancing. Figure 1A illustrates the first three responses in phase contrast micrographs from three different turning assays. The position of the growth cone at the end of the 60-min assay period in a ‘control’ gradient deviated only slightly from the initial direction of growth (second panel, Fig. 1A). In response to an ‘attractant’ (HGF) the growth cone in the third panel (Fig. 1A) moved from its initial position to a point close to the micropipette, whereas the growth cone in the last panel moved away from a micropipette releasing the ‘repellent’ thrombin. Corresponding rosebud plots show the paths of several growth cones in Fig. 1B. These plots also illustrate the considerable variations in turning and growth rate responses inherent in the experiments. Cumulative distribution plots of final turning angles are shown in Fig. 1C. The shift in turning angle distribution, from repulsion by thrombin to attraction by HGF, is clearly evident, with control angles more or less symmetrically distributed on either side of 0° and IGF-1 slightly (but not significantly) shifted toward attraction. Results from these (and all subsequent) experiments were analysed with the Kolmogorov–Smirnov goodness-of-fit test to determine whether data were normally distributed. All of our data passed the test so that they could be subjected to parametric statistical analysis.

Final turning angles for DRG growth cones responding to seven different factors are shown in Fig. 2A and listed in Table 3. Both HGF and NPY elicited strong attraction, with final turning angles of

![Fig. 2](image-url)

(A) Final turning angles (means ± SEM) in response to gradients of different factors. Student’s t-tests were performed to determine significance relative to control (n values for each condition shown on right). pCEP4 is the control for semaphorin 3A (Sema3A). Factors without P-values did not elicit statistically significant changes. (B) Neurite growth rates in response to different factors (gradient application), expressed as μm/h (means ± SEM). Measurements were taken at 30 and 60 min for the same sets of neurites. These values were essentially the same. Student’s t-tests were performed to determine significance of change relative to control (n values for each condition shown below factors). *P ≤ 0.03; †P ≤ 0.008. HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor; NGF, nerve growth factor; NPY, neuropeptide Y.
Because we wanted to assess growth cone turning and advancement under identical conditions, growth rates were measured in gradients of the different factors described above. Thus, these assays were different from those using a constant factor concentration applied uniformly in the culture medium (‘bath application’). While the factor concentrations at the growth cones changed with their advance, the gradients also should be noted that the gradient paradigm may resemble the culture medium (‘bath application’). While the factor concentrations at the growth cones changed with their advance, the gradients also should be noted that the gradient paradigm may resemble the culture medium (‘bath application’). While the factor concentrations at the growth cones changed with their advance, the gradients also should be noted that the gradient paradigm may resemble the culture medium (‘bath application’). While the factor concentrations at the growth cones changed with their advance, the gradients also should be noted that the gradient paradigm may resemble the culture medium (‘bath application’). While the factor concentrations at the growth cones changed with their advance, the gradients also should be noted that the gradient paradigm may resemble the culture medium (‘bath application’). While the factor concentrations at the growth cones changed with their advance, the gradients also should be noted that the gradient paradigm may resemble the culture medium (‘bath application’). While the factor concentrations at the growth cones changed with their advance, the gradients also should be noted that the gradient paradigm may resemble the culture medium (‘bath application’).

### Growth cone advancement

Growth rate increases

<table>
<thead>
<tr>
<th>Factor</th>
<th>Type</th>
<th>Final turning angle</th>
<th>Growth rate increase over control (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGF</td>
<td>GF</td>
<td>23.5°S</td>
<td>1.9 S</td>
</tr>
<tr>
<td>NPY</td>
<td>NP</td>
<td>15.1°S</td>
<td>1.9 S</td>
</tr>
<tr>
<td>NGF</td>
<td>GF</td>
<td>7.2°S</td>
<td>1.7 S</td>
</tr>
<tr>
<td>IGF-1</td>
<td>GF</td>
<td>5.2°S</td>
<td>1.9 S</td>
</tr>
<tr>
<td>Galanin</td>
<td>NP</td>
<td>1.7°</td>
<td>1.9 S</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>−0.6°/−0.6**</td>
<td>1</td>
</tr>
<tr>
<td>Sema3A</td>
<td>R</td>
<td>−13.2°S</td>
<td>0.9</td>
</tr>
<tr>
<td>Thrombin</td>
<td>R</td>
<td>−22.8°S</td>
<td>2.0 S</td>
</tr>
</tbody>
</table>

GF, growth factor; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor-1; NGF, nerve growth factor; NP, neuropeptide; NPY, neuropeptide Y; R, repellent; S, significantly different from its control (for Sema3A, pCEP4); Sema3A, semaphorin 3A. *Values for standard medium control/pCEP4 control relevant to Sema3A (growth rates for both controls set to 1).

NPY was less pronounced (65.4 ± 5.2 μm/h; P ≤ 0.03). Relative to control, pCEP4 (the control for Sema3A) resulted in an apparent increase in growth rate of about 40%, but this was not statistically significant (P ≤ 0.08). Our Sema3A results fell in between control and pCEP4, and were not significantly different from either, so that Sema3A did not affect the growth rate in these experiments. For juxtaposition with turning angles, growth rate increases are listed in Table 3.

### Effects of factor combinations

NPY has been shown to inhibit the collapse response of growth cones to repellents, specifically Sema3A (Tuttle & O’Leary, 1998; Dontchev & Letourneau, 2002). However, the ability of NFG to modulate the response of growth factors and attractants has been the subject of controversy. In our particular experiments, we wanted to examine the growth cone’s responses to attractants and growth factors in the presence of NFG. For the experiments described further above, DRG neurons were plated first in culture medium supplemented with NFG and, 24 h later, switched to NFG-free medium. For the present experiments, however, the DRG neurons were kept in NFG-containing medium at the same NFG concentration. Under these conditions, growth rates are essentially the same as those observed without NFG present during the assays (Fig. 3A). We performed turning assays for two factors, IGF-1 and HGF, in this medium (see Table 2). By itself, HGF was both a strong growth promoter and attractant, and IGF-1 was a strong growth promoter but not a significant attractant (Fig. 3A and B). Interestingly, in the presence of NFG, growth rates in gradients of both HGF and IGF-1 not only were reduced to a level well below that of each factor alone (HGF, P ≤ 0.001; IGF-1, P ≤ 0.0002), but also dropped below those measured for bath application of NFG alone (HGF, P ≤ 0.03; IGF-1, P ≤ 0.003). In other words, the presence of NFG turned HGF- and IGF-1-elicted growth promotion into inhibition. Likewise, the attractant effect of HGF was neutralized by NFG (P ≤ 0.01). NFG also seemed to abrogate what appeared to be weak IGF-1-induced turning, but this change was not significant statistically (P = 0.1). (Bath application of NFG, without factor gradient, cannot elicit a turning response and, therefore, is not shown.)

### Effects of Y1 receptor antagonist

NPY is the most abundant neuropeptide in the mammalian nervous system and can signal through several G-protein-coupled receptors (Y1, Y2, Y4 and Y5; Larhammar, 1996; Lundberg, 1996; Lundberg et al., 1996; Pedrazzini et al., 2003; Silva et al., 2005). DRG are known to express at least two of the receptors, Y1 and Y2 (Brumovsky et al., 2007). Having established that NPY was a growth promoter as well as an attractant for DRG neurons, we were interested in determining whether the signaling for turning and growth was regulated through one or more receptors.

We performed turning assays to NPY in the presence of an Y1 receptor antagonist, H 409/22 (Malmstrom et al., 2000). H 409/22 is a non-peptide antagonist with a similar structure to that of the first highly potent and selective Y1 receptor antagonist BIBP32261 (Rudolf et al., 1994). It possesses a high affinity for rat and human Y1 receptors with an IC value of 16 ± 3 nM for rat brain cortex receptors, and it is devoid of affinity for Y2, Y4 and Y5 receptors (Bergman et al., 1999; Gedda et al., 1999). The antagonist alone did not affect growth rates (Fig. 4A). However, it completely blocked the ability of NPY to increase the rate of extension (Fig. 4A). The receptor antagonist dropped the growth rate for NPY from 65.4 ± 5.2 μm/h to 35.8 ± 8.4 μm/h (P ≤ 0.002). Compared with H 409/22 alone, NPY gradient application in the presence of the antagonist did not significantly change growth rates. Furthermore, the Y1 antagonist
reduced the NPY-elicited turning angle from 15.1 ± 5.0° to 3.0 ± 6.8° (P ≤ 0.02; Fig. 4B). Thus, H 409/22 diminished growth rates and final turning angles to control levels. It follows that NPY elicits both of its growth cone responses through the Y1 receptor.

Fig. 3. Effects of factor combinations on growth rates and final turning angles. (A) Growth rates of DRG neurites in control medium (from Fig. 2B) vs. nerve growth factor (NGF)-containing medium, in response to gradient application of hepatocyte growth factor (HGF) and insulin-like growth factor-1 (IGF-1; mean μm/h ± SEM). Values for n and P are shown below conditions. (B) Turning responses (mean final turning angles ± SEM) to HGF or IGF-1 gradients, with or without NGF present (n values for each condition are indicated on the right). In both (A) and (B) significance was determined using Student’s t-test.

Fig. 4. Effects of Y1 receptor antagonist, H 409/22, on growth cone advance and turning in neuropeptide Y (NPY) gradients. (A) Growth rates in response to NPY (gradient application), in the presence or absence of H 409/22 (mean μm/h ± SEM). (B) Growth cone turning in response to NPY, in the presence or absence of H 409/22 (mean final turning angles ± SEM). In both (A) and (B) significance of change relative to NPY alone was determined using Student’s t-test (n values are indicated for each condition).

Discussion

The majority of data available on growth cone responses to various factors [BDNF, neurotrophin-3 (NT-3), netrin-1, Slit, agrin, bone morphogenic protein and ACh] come from Xenopus spinal neurons (Zheng et al., 1994; Song et al., 1997; Stein & Tessier-Lavigne, 2001; Xu et al., 2005; Wen et al., 2007). Results indicate that, for some factors (netrin, BDNF; Stein & Tessier-Lavigne, 2001), attraction/growth promotion and repulsion/growth inhibition may
be correlated, whereas for others (BDNF, NT-3, agrin, ACh; Zheng et al., 1994; Song et al., 1997; Xu et al., 2005) they may not be linked. More disturbingly, the two sets of BDNF data (Song et al., 1997; Stein & Tessier-Lavigne, 2001) are inconsistent, and an agrin gradient causes repulsion without affecting axonal growth rate, while bath application of agrin reduces growth rates (Xu et al., 2005). The situation is clearer for netrin, which seems to be an attractant and growth promoter, and for Slit, which seems to be a ‘pure’ growth promoter (Stein & Tessier-Lavigne, 2001). The latter suggests that attraction and growth promotion may be independent, at least for Slit in Xenopus spinal neurons.

The most commonly used assay to evaluate neurite growth is to culture explants in specific medium conditions for extended periods of time (many hours) and then to measure the overall length of the radial outgrowth. Results from such semi-quantitative assays have been used to calculate growth rates (Levi-Montalcini et al., 1954; Lumsden & Davies, 1983; Tessier-Lavigne et al., 1988), but the values obtained tend to be derived from the longest (but unidentified) neurites of heterogeneous bundles, and they do not take into account: (i) the time between establishment of the culture and initiation of outgrowth; (ii) adaptive effects (such as receptor downregulation); (iii) possible collapse and retraction of neurites; and (iv) long-term effects caused by changes in gene expression. In contrast, our assays monitored individual, established neurites, previously grown for at least 1 h in control conditions (no NGF), in the presence or absence of specific factors for 60 min only. Tracking the growth cones of these neurites ascertained that measurements came from neurites that were advancing continuously. This provided true growth rates in response to the factors, without interference by longer-term effects. Indeed, growth rates measured over 30 min were equal to those obtained from the same neurites at 60 min. This indicated that even if desensitization occurred in our gradient assays, it did not affect growth rates.

A number of studies on growth cone turning responses are based on co-culture assays involving explants of neural tissue in the vicinity of cell aggregates expressing a factor of interest (Levi-Montalcini et al., 1954; Lumsden & Davies, 1983; Tessier-Lavigne et al., 1988). These assays provide excellent qualitative data on the behavior of a growth cone population in a factor gradient. However, factor concentrations are unknown, and assays are difficult or impossible to quantify. The number of reports on mammalian growth cone guidance in defined gradient conditions is very limited (Xiang et al., 2002; Ding et al., 2007), and growth and turning responses have not been analysed systematically in the same system and the same conditions. This was the main reason for undertaking the present study.

In order to summarize and display our growth cone data we have generated the scatter plot shown in Fig. 5. The abscissa shows growth rates, whereas the ordinate indicates turning angles. This allows one, for example, to look for correlation between the parameters. An intuitive hypothesis might have been that correlations exist between attraction and growth promotion, or between repulsion and growth reduction. In that case one would expect the data points to define a diagonal line running from strong repulsion/low growth rate (lower left), through control, to strong attraction/high growth rate (upper right). However, as the plot makes immediately clear, these correlations do not exist.

**Turning and growth promotion**

The amplitudes we measured for the turning responses of DRG growth cones are comparable to those obtained for Xenopus spinal neurons, but the growth rates of the Xenopus axons are several fold lower than the ones we describe here. Growth cone turning involves asymmetric filopodial and lamellipodial protrusion (Gallo & Letourneau, 2002), and translocation (i.e. disassembly and re-assembly in a new location) of adhesion sites (Gatlin et al., 2006). To become detectable, therefore, a turning response must be accompanied by a minimum amount of growth cone advance. Indeed, robust turning responses in our experiments generally were accompanied by a substantial increase in growth rate. Surprisingly, this also applied to the strong repellent, thrombin (at a sub-collapse concentration), which was as potent a growth promoter as IGF-1, galanin or HGF. Sema3A, however, did not evoke an increase in growth rate, and growth cones continued to advance at about 54 μm/h (not significantly different from its control, pCEP4). Sema3A has been reported to inhibit axonal outgrowth (Togashi et al., 2006; Ben-Zvi et al., 2007). However, these studies assessed overall outgrowth from explants after extended periods of culture, without concern for collapse or retraction events that may have influenced the results. Thus, it is not clear whether axonal growth rates changed in those experiments. It should be kept in mind that uniform and high concentrations of repellent lead to growth cone collapse and, thus, cessation of advancement. However, our repellent experiments were designed so as to elicit a clear-cut turning response without collapse.

One might predict that asymmetric application (as a gradient) of an outgrowth-promoting factor (such as galanin or IGF-1) would prompt a turning response because portions of the growth cone near the source of factor would receive a stronger stimulus. As the examples of galanin and IGF-1 show, however, this is not the case. Thus, ‘pure’ promoters of neurite advance exist for mammalian neurons (see also Slit in Xenopus; Stein & Tessier-Lavigne, 2001). As a corollary, growth cone turning and advance must be mechanistically distinct.

**Fig. 5.** Scatter plot of growth cone responses to gradients of seven different factors. Responses to insulin-like growth factor-1 (IGF-1) and hepatocyte growth factor (HGF) gradients in the presence of nerve growth factor (NGF), and to neuropeptide Y (NPY) gradients in the presence of H 409/22 are shown also. Final turning angles are represented by the ordinate, with the dark horizontal line at 0° deflection; growth rates in μm/h are shown by the abscissa, with the dark vertical line marking control growth rate. Sema3A, semaphorin 3A.
Growth cone turning and advance are regulated independently

One of the most surprising results of our studies is the broad scatter of data points seen in Fig. 5 (as opposed to the diagonal mentioned above). This indicates that, aside from a minimal growth requirement, there is no correlation between turning and growth responses (see also Table 3). This is illustrated best by the following examples: (i) strong growth promoters may be attractants or repellents (such as HGF vs. thrombin); and (ii) equally strong growth promoters may or may not elicit an attractive response (HGF vs. galanin or IGF-1, respectively). However, independence of growth and turning seems to be restricted in one way: none of the factors tested elicited a strong turning response without continued growth cone advance. In Fig. 5, such a factor would have generated a point significantly above or below a line connecting control to HGF or thrombin, respectively. We have not found an example of such a factor in the literature either. It follows that growth cone advance and turning are regulated independently, but that a strong turning response typically seems to be correlated with strong growth promotion.

Upon cursory examination in vitro, growth cone turning may be described as asymmetric advance. However, our data show that of two strongly growth-promoting, asymmetrically applied factors, IGF-1 and HGF, only HGF triggers an attractive response. Therefore, growth cone attraction cannot be explained simply by asymmetric growth promotion. There must be significant mechanistic differences. It has been known for some time that growth cone advance necessitates an optimal cytosolic Ca$^{2+}$ level (Kater et al., 1988; Henley & Poo, 2004). A role for Ca$^{2+}$ has been invoked for growth cone turning as well, but the situation seems to be considerably more complex. It has been demonstrated that localized, asymmetric Ca$^{2+}$ transients in the growth cone are sufficient to cause turning, but the response depends on the resting Ca$^{2+}$ level: at higher levels the growth cone turns to the side of the transients, at lower levels away from them (Zheng, 2000; Henley & Poo, 2004). Indeed, it has been demonstrated that gradients of a number of attractant factors elicit Ca$^{2+}$ transients on the side of the growth cone proximal to the source of the factor and/or require Ca$^{2+}$ signaling (BDNF, netrin-1, BMP7; Song et al., 1997; Hong et al., 2000; Wen et al., 2007). However, Ca$^{2+}$ signaling also is involved in the action of the repellent, myelin-associated glycoprotein (Henley & Poo, 2004). Yet, signaling of the archetypal repellent, Sema3A, apparently does not involve Ca$^{2+}$ transients (Song et al., 1998; Shim et al., 2005). Together these reports suggest that the roles of Ca$^{2+}$ in neurite extension and turning are different. This is perhaps best exemplified by the observation that reduction of extracellular Ca$^{2+}$ from 1 mM to 1 μM blocks attraction by BDNF but increases the rate of neurite extension in Xenopus neurons (Song et al., 1997). Hence, studies on Ca$^{2+}$ support the observation that neurite extension and turning are mechanistically distinct.

Conceivably, regulation of turning and advance could occur through different receptors for the same factor. For example, NPY is known to potentially interact with at least four different receptors (Larhammar, 1996; Pedrazzini et al., 2003; Silva et al., 2005). However, inhibition of the Y1 receptor with the highly selective H409/22 (Bergman et al., 1999; Gedda et al., 1999) blocks both the growth and turning responses to NPY. For some of the factors we tested, such as HGF and Sema3A, only one receptor is known (c-Met; neuropilin-1; Bottaro et al., 1991; He & Tessier-Lavigne, 1997; Kolodkin et al., 1997) so that these factors’ pleiotropic effects also must be mediated through single receptors. Likewise, the attraction and growth promotion observed for BDNF and netrin in Xenopus are mediated through single receptors (Stein & Tessier-Lavigne, 2001). It follows that the degree to which a factor influences growth cone turning vs. advance is controlled at a branch point downstream in the receptor-activated pathway (e.g. gradient application of galanin, growth promotion only; HGF, growth promotion plus turning).

Neuropeptides

Neuropeptides represent the largest group of signaling molecules in the nervous system with a correspondingly large number of receptors. They may have both transmitter- and growth factor-like effects as well as other functions. With regard to the two molecules studied here, NPY and galanin, the former has been shown to promote neurite elongation (White, 1998) and to induce proliferation of neurons in the olfactory mucosa via the Y1 receptor (Hansel et al., 2001). NPY is expressed in the ensheathing cells (Doucette, 1990) around the olfactory nerves (Ubink et al., 1994), which support growth of olfactory axons throughout life (Graziadei & Monti Graziaide, 1978). To test the hypothesis that NPY has a guidance function, NPY $\rightarrow$ ~ mice (Erickson et al., 1996) were crossed with mice in which a single olfactory receptor was genetically labeled with a tau-LacZ construct (Mombaerts et al., 1996). However, no defect in olfactory neuron pathfinding could be revealed (Ubink et al., 2003). In contrast, using a DRG neuron model, we show here that NPY not only has a growth-promoting effect, as shown by White (1998), but also induces a strong turning response, associated with the NPY Y1 receptor.

Interestingly, in adult rats and mice NPY is expressed in DRG cells only after axotomy, but not in intact DRG neurons (Wakasaka et al., 1991; Corness et al., 1996). Pancreatic peptide YY, but not NPY, is transiently expressed in DRGs at embryonic day 16 (Jazin et al., 1993), and this peptide acts on Y1 receptors. Thus, activation of the Y1 receptor in DRG neurons may influence neurite outgrowth and pathfinding in two critical situations, during development and after nerve injury.

With regard to galanin, it is now well established that this peptide exerts trophic actions on neurite outgrowth, including that from DRG neurons, mediated via the GalR2 receptor (Mahoney et al., 2003; Hobson et al., 2006). This is in agreement with our findings of growth stimulation.

NGF can inhibit both attraction and growth promotion

The inhibition of growth cone repulsion and collapse by NGF has been known for some time (Tuttle & O’Leary, 1998; Donetchev & Letourneau, 2002). In contrast, the literature regarding NGF modulation of growth factor or attractant effects is inconsistent. NGF and IGF-1 in combination have been reported to affect neurite outgrowth of adult DRG synergistically (Jones et al., 2003) or not at all (Kimpinski & Mearov, 2001). NGF combined with BDNF inhibits neurite extension in adult rat DRGs (Kimpinski et al., 1997). In contrast, when DRG neurons are plated and grown in medium containing both NGF and HGF, HGF enhances the neurite outgrowth seen with NGF alone (Maina et al., 1997). Cross-talk between attractants has been reported as well: Xenopus growth cones that were preincubated with either one of two attractants, netrin-1 or BDNF, and then exposed to a gradient of the other attractant lost their ability to detect these gradients, and this adaptation disappeared after a re-sensitization period (Ming et al., 2002).

It was a surprising finding, therefore, that the combined effects of NGF (bath) and growth factors/attractants (gradient) were not additive but inhibitory in our experiments. In the presence of NGF, HGF’s
ability to attract the growth cone was neutralized, and both IGF-1 and HGF caused a reduction rather than an increase in growth rates relative to NGF-only control. There are at least two possible, not mutually exclusive, explanations for NGF inhibition of attraction. Firstly, the growth cones may have been unable to respond to a gradient because NGF uniformly present in the culture medium stimulated them from all directions. Alternatively, NGF in the medium may have downregulated Trk-A-activated signaling components shared with the IGF-1 or HGF receptor pathways, resulting in adaptation (see also Paves & Saarma, 1997; Stein & Tessier-Lavigne, 2001; Ming et al., 2002). Finally, the action of both factors could have raised cytosolic Ca2+ to an inhibitory level (see Discussion above; Zheng, 2000; Henley & Poo, 2004). Jones et al. (2003) showed that, at least in adult rat neurons, intracellular signals evoked by IGF-1 and NGF converged onto the phosphoinositide-3-kinase-Akt-glycogen synthase kinase-3 pathway, a potential site of adaptation. There are several possible explanations for the discrepancy between the findings of Jones et al. (2003) and ours: (i) the developmental ages of the neurons were different in the two cases (adult vs. fetal), and signaling of neurotrophins may change with development (Markus et al., 2002); (ii) rather than performing stimulation assays, as in our experiments, Jones and colleagues plated and then maintained the neurons with both factors present; and (iii) the analytical methods were different in that Jones et al. (2003) assessed outgrowth at the end of a long period, whereas we measured growth rate over 60 min using time-lapse microscopy. Overall, our finding that uniformly applied NGF neutralizes attraction elicited by HGF may be explained by a desensitization/adaptation phenomenon (Ming et al., 2002). In contrast, the observations that HGF and IGF-1 are growth inhibitory rather than growth promoting in the presence of NGF are more difficult to explain but may be related to Ca2+ homeostasis in the growth cone.

Summary and conclusions

Our observations demonstrate that growth cones respond to different factors with diverging degrees of turning and growth enhancement, and that turning and advance are regulated largely independently, with the exception that strong turning responses of either type typically seem to correlate with strong growth promotion. Notably, asymmetric gradient application of growth-promoting factor does not per se elicit an attractive response. Data indicate that the growth and guidance factors’ pleiotropic effects, at least in some cases, are mediated through a single receptor. Finally, the pathways activated by different receptors interact, as is evident from the observation that uniformly applied NGF inhibits not only repellent (Tuttle & O’Leary, 1998; Döntchev & Letourneau, 2002) and attractant responses, but also turns growth promotion elicited by IGF-1 or HGF into inhibition. These results are also important because they define, for the first time, the growth cone responses of a single identified mammalian neuron to a broad spectrum of factors. Such data are a prerequisite for further studies on the complex regulatory networks that control the growth cone’s machinery for pathfinding.

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Abbreviations

ACH, acetylcholine; BDNF, brain-derived neurotrophic factor; DRG, dorsal root ganglia; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor-1; NGF, nerve growth factor; NPY, neuropeptide Y; NT-3, neurotropin-3; Sema3A, semaphorin 3A.

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