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Endocytosis-dependent desensitization and protein synthesis–dependent resensitization in retinal growth cone adaptation

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Abstract

It has been proposed that growth cones navigating through gradients adapt to baseline concentrations of guidance cues. This adaptation process is poorly understood. Using the collapse assay, we show that adaptation in *Xenopus laevis* retinal growth cones to the guidance cues Semaphorin 3A or netrin-1 involves two processes: a fast, ligand-specific desensitization that occurs within 2 min of exposure and is dependent on endocytosis, and a slower, ligand-specific resensitization, which occurs within 5 min and is dependent upon protein synthesis. These two phases of adaptation allow retinal axons to adjust their range of sensitivity to specific guidance cues.

The continuous receptor-mediated signaling that occurs when a cell interacts with its environment can be regulated by adaptation. From the immune system to the nervous system, the result of the adaptation process is usually a resetting of sensitivity^{1,2}. Adaptation seems to be especially crucial for the chemotropic responses of cells, including bacteria³ and macrophages^{4,5}, in gradients of attractants or repellents. It seems reasonable to expect that adaptation also has a role in the chemotropic responses of growth cones. Previous work has shown that retinal growth cones launched on a platform of a repulsive guidance factor could grow further up an increasing gradient of the factor than could growth cones that were not launched on the platform, demonstrating that adaptation can extend the sensitivity of axons to guidance cues⁶. This adaptation process could be used for axonal orientation in gradients of guidance molecules, such as the ones retinal axons encounter in the tectum. Others have argued that adaptation may also be important along the pathway where axons meet various guidance cues and might need to adjust their sensitivity to navigate correctly⁷.

Adaptation is often associated with a fast desensitization response and a slower resensitization response⁸. The phenomenon of growth cone desensitization was first demonstrated in chick retinal growth cones that failed to respond to a collapse-inducing signal after it was repeatedly presented^{9,10}. Desensitization has also been shown to be involved in enabling growth cones to move on from attractive intermediate targets. Netrin-1 attracts commissural axons to the midline of the spinal cord, yet once these axons are exposed to netrin-1 they lose responsiveness to it¹¹. Desensitization and resensitization have also been described in embryonic *X. laevis* spinal growth cones exposed to attractants¹². Exposure to low levels of an attractant (either BDNF or netrin-1) for 30 min caused growth cones to fail to turn towards a gradient of this factor, but if the exposure was continued for another 30–60 min, the growth cones recovered their ability to respond.

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Are the sequential processes of desensitization and resensitization part of a homeostatic reset mechanism that allows a growth cone to transiently turn its response off, then on again, so that it can sense the same guidance cue in the same way if it is presented a second time? Or do these processes allow a growth cone to adjust its sensitivity appropriately to particular background levels of a guidance cue? Here, we report on the cellular mechanisms underlying desensitization and resensitization in retinal growth cones, which allow them to adjust their sensitivity down and up as a function of exposure to a specific ligand.

RESULTS

Rapid desensitization and resensitization in growth cones

We investigated the time course of desensitization and resensitization of retinal growth cones to guidance cues using collapse assays. We chose collapse assays because they are rapid, enabling the repulsive chemotropic responses of large numbers of growth cones to be monitored within minutes. *X. laevis* retinal growth cones undergo collapse in response to Semaphorin 3A, detectable within 2 min of exposure and maximal at 10 min¹³. Collapse is probably related to repulsive turning responses, as guidance cues that elicit repulsive turning when presented in a gradient usually cause collapse when added uniformly. The difference between a repulsive turn and collapse may, therefore, simply be the difference between a polarized and a global response. Such assays allowed us to measure the time course of desensitization and resensitization accurately (as shown in Fig. 1a). We exposed growth cones to a low dose of a guidance molecule that produced a minimal amount of growth cone collapse for various periods of time. This was immediately followed by exposure to a high dose that consistently produced a maximal collapse response.

A pretreatment time of 2 min to a low dose of either Semaphorin 3A or netrin-1 caused a marked decrease in collapse in response to the high dose of either molecule, demonstrating that growth cones become rapidly desensitized (Fig. 1b). To investigate the time course of this effect more fully, pretreatment times were varied from 30 s to 30 min. Pretreatment with a low dose of Semaphorin 3A resulted in desensitization within 90 s, with maximal desensitization at 2 min. Growth cones then showed steady resensitization, showing a maximal response to a high dose after 4 min (Fig. 1b). Netrin-1 produced even faster desensitization, with growth cones being maximally insensitive after only 1 min of low-dose pretreatment and becoming resensitized within 5 min (Fig. 1b). Pretreatments for times ranging from 10–30 min did not show any further significant changes, suggesting that the resensitization process is complete by 5 min.

Recalibration or homeostatic reset?

What is the function of growth cone desensitization and resensitization? Is it a homeostatic reset mechanism, whereby a growth cone that collapses in response to a dose of a guidance factor is able to recover from this encounter by first disengaging from the collapse-inducing factor and then re-engaging its response elements so that the same dose of a collapse factor can subsequently lead to the same response? Or are the processes of desensitization and resensitization used to adjust the sensitivity of a growth cone so that when a growth cone is exposed to a particular background level of a guidance cue, it adjusts or recalibrates its sensitivity? This latter recalibration mechanism is what is known, in the field of bacterial research, as adaptation, as it allows bacteria to climb up concentration gradients of attractants. If adaptation in retinal growth cones is a recalibration process, then a growth cone exposed to a background level of a repellent such as Semaphorin 3A should need more Semaphorin 3A than a naïve growth cone to cause the same collapse response, and such exposed growth cones should respond differentially to larger amounts of Semaphorin 3A that unexposed growth cones could not differentiate: that is, the dose-response curve should be shifted and

extended to higher doses in adapted as compared to naive growth cones. To test this idea directly, we compared the responses to a variety of doses of *Sema3A* in collapse assay of (i) naive growth cones and (ii) growth cones exposed to a low-dose pretreatment with *Sema3A* for 5 min (allowing resensitization). Naive growth cones showed more collapse than growth cones exposed to the low-dose pretreatment at all doses of *Sema3A* except the high dose (Fig. 2). Furthermore, naive growth cones also seemed to saturate their response at lower doses than exposed growth cones, showing that exposed growth cones can distinguish these higher doses, and suggesting that exposure to a low dose shifts the dose-response curve towards higher levels of the collapsing factor (Fig. 2). These results support a model in which desensitization and resensitization are components of an adaptation process that is involved in adjusting the sensitivity of growth cones in a way that could enable them navigate in gradients of guidance cues^{6,7}.

Resensitization requires protein synthesis

To determine whether protein synthesis is involved in desensitization or resensitization, we blocked protein synthesis during the low-dose exposure by adding cycloheximide, a reversible inhibitor of the protein translocation reaction on ribosomes. At the end of all pretreatments involving pharmacological reagents, cultures were rinsed in inhibitor-free medium (see Methods). Then a high dose of *Sema3A* or netrin-1 was added in the absence of cycloheximide. The presence of cycloheximide during low-dose pretreatment did not alter desensitization of growth cones to either guidance cue (Fig. 3a,b). However, resensitization of growth cones did not occur when protein synthesis was inhibited during pretreatment (Fig. 3a,b). This implies that retinal growth cone resensitization, but not desensitization, is dependent on local protein synthesis. Inhibition of growth cone resensitization was also observed when the reversible inhibitor of peptidyltransferase activity on the ribosomes, anisomycin, was added during the pretreatment period (Fig. 3c). The rapid reversibility of cycloheximide and anisomycin is critical for these experiments, and is shown by the fact that axons pretreated with either cycloheximide or anisomycin alone, for various periods, show full collapse when exposed to a high dose of either netrin-1 or *Sema3A*, whereas exposure of growth cones to these guidance cues in the presence of cycloheximide or anisomycin prevents collapse¹⁴. The findings support the hypothesis that protein synthesis is required for growth cone resensitization¹².

Desensitization is dependent on endocytosis

As desensitization of retinal growth cones was not dependent on protein synthesis, a different cellular mechanism must underlie this aspect of adaptation. *Sema3A*-induced collapse of neuronal growth cones is accompanied by increased endocytosis^{15,16}, and the *Sema3A* receptors neuropilin-1 and plexin colocalize with endocytic vacuoles after *Sema3A* stimulation¹⁶. To test whether desensitization by *Sema3A* or netrin-1 is dependent on endocytosis, growth cones were pretreated with a low dose of either *Sema3A* or netrin-1 in the presence of phenylarsine oxide (PAO), an inhibitor of receptor-mediated endocytosis^{5,17}. Pretreatment of cultures with a low dose of *Sema3A* or netrin-1 and PAO clearly inhibited desensitization (Fig. 4a,b), illustrating that receptor-mediated endocytosis may be an essential step in growth cone desensitization.

Multiple endocytic pathways are used in biological systems. Clathrin-mediated endocytosis targets proteins to the endosomal pathway, whereas lipid raft and caveolar pathways act as alternative means of trafficking¹⁸. As PAO nonspecifically inhibits endocytosis, we used a more selective reagent. MDC is a competitive inhibitor of transglutaminase, an enzyme essential for the formation of clathrin-coated vesicles^{19,20}. Pretreatment with MDC and a low dose of either molecule also suppressed growth cone desensitization (Fig. 4c),

suggesting that endocytosis induced by Sema3A and netrin-1 occurs in a clathrin-dependent fashion.

Endocytosis of receptors and desensitization

Neuropilin-1, a transmembrane glycoprotein that interacts with Sema3A^{21,22}, forms a receptor complex with another transmembrane protein, plexin A1 (ref. 23). Binding of Sema3A to neuropilin-1 causes the dissociation of plexin from the receptor complex and initiates second-messenger pathways^{24,25}. We therefore wanted to know if the endocytic removal of neuropilin-1 from the cell surface mediated desensitization to Sema3A. To investigate this, we exposed cultures to a low dose of Sema3A for times ranging from 2 to 10 min and subsequently analyzed the abundance of cell surface neuropilin-1 on growth cones by quantitative immunohistochemistry, using an antibody against the extracellular domain of neuropilin-1 in nonpermeabilized conditions (Fig. 5). Addition of a low dose of Sema3A for 2 min significantly diminished the amount of cell surface neuropilin-1 present on axonal growth cones (Fig. 5a–e). After 5 min treatment, cell surface neuropilin-1 reactivity had returned to levels seen before stimulation. Thus, the time course for removal and replacement of neuropilin-1 matches that course for desensitization and resensitization. Similar experiments were performed using an antibody to the extracellular domain of DCC after exposure to a low dose of netrin-1. Again, the removal and replacement of DCC from the surface of the growth cone mirrored the time course for desensitization and resensitization (Fig. 6a–e).

We next assessed whether surface receptor loss was dependent on endocytosis by applying inhibitors. Indeed, PAO abolished Sema3A-induced neuropilin-1 depletion at the cell surface (Fig. 5f,i–k). Similar results for the netrin-1 receptor, DCC (Fig. 6f,i–k), suggest that receptor endocytosis in growth cones may mediate desensitization.

Replacement of receptors and protein synthesis

Because desensitization was correlated with the endocytosis of receptors at the cell surface, we wondered whether the reappearance of receptors at the cell surface was dependent on protein synthesis during resensitization. We therefore exposed growth cones to a low dose of Sema3A + cycloheximide. A 2-min exposure showed that neuropilin-1 removal from the cell surface (Fig. 5g,k) was unaffected, which means that protein synthesis is not involved in receptor removal. What was more interesting was the reappearance of at least some of the receptor after 5 min of treatment with a low dose of Sema3A + cycloheximide (Fig. 5h,k).

Similar experiments were performed after growth cone exposure to a low dose of netrin-1 + cycloheximide. Removal of DCC from the growth cone surface was not dependent on protein synthesis (Fig. 6g,k). In this case, too, we observed a partial recovery of DCC to the growth cone surface after 5 min of treatment with a low dose of netrin-1 + cycloheximide (Fig. 6h,k). These results suggest that protein synthesis may be required for the reappearance of receptors on the growth cone surface. However, the partial recovery of cell surface receptor in the absence of protein synthesis suggests that some of the reappearance is due to recycling from the endosomal compartment back to the plasma membrane.

Desensitization and resensitization are ligand specific

To test whether the adaptation process was ligand specific, we carried out collapse assays in which cultures were pretreated with a low dose of Sema3A and then treated with a high dose of netrin-1, or vice versa. Pretreatment with a low dose of Sema3A for 2 min did not cause desensitization to a high dose of netrin-1, and similarly, pretreatment with a low dose of netrin-1 (2 min) did not desensitize growth cones to a high dose of Sema3A (Fig. 7a). These data suggest that adaptation is ligand specific and are consistent with the idea that the loss of

receptor from the surface mediates desensitization. Resensitization is protein synthesis dependent, whereas receptor replacement is only partially dependent on protein synthesis. Therefore, resensitization may rely, at least in part, on the synthesis of molecules that are common to both the *Sema3A* and *netrin-1* pathways. For example, it could be that increased translation of cytoskeletal proteins such as β -actin could be involved in resensitization. If so, then resensitization should not be ligand specific. To test this, we performed a 5-min low-dose pretreatment with one molecule to allow complete resensitization and then tested with the other molecule. If there were cross-resensitization, we would expect a higher than normal collapse rate. We found no evidence of cross-resensitization (Fig. 7b), which suggests that resensitization, like desensitization, may also be ligand specific (Fig. 7b). In this experiment, however, it is possible that the collapse response was saturated. To address this potential difficulty, we used an intermediate dose rather than a high dose of *Sema3A* after conditioning the growth cone to a low dose of *netrin-1*. The intermediate dose of *Sema3A* caused submaximal collapse in naive neurons, and this was not significantly altered in growth cones adapted to a low dose of *netrin-1*: that is, there is no indication of cross-resensitization (Fig. 7c). That both phases of adaptation are ligand specific implies that growth cones can fully adapt to one ligand, or family of related ligands, without changing their sensitivity to other ligands.

DISCUSSION

We suggest that desensitization and resensitization are two phases of an adaptational mechanism that can be used to reset the sensitivity of growth cones as they grow through increasing concentration of ligands, such as the gradients of ephrins in the tectum, or along the pathway as they navigate in an epithelium that exposes them to a multitude of different guidance factors at different concentrations. This suggestion is based on findings that retinal growth cones pretreated with a low dose of a repellent showed a rapid, endocytosis-dependent desensitization in response to a high dose in a collapse assay. Resensitization was also very rapid, with growth cones regaining full responsiveness to collapse within 5 min of pretreatment. Protein synthesis was essential for resensitization. We also found that neuropilin-1 and DCC, the *Sema3A* and *netrin-1* receptors, undergo an endocytosis-dependent depletion from the growth cone surface within minutes of exposure to their respective guidance cue, followed by a partially protein synthesis-independent reinsertion into the plasma membrane of the growth cone within 5 min of exposure. Finally, we found that these responses are ligand specific and that exposure to a low dose of *Sema3A* shifts the sensitivity of growth cones to higher doses of *Sema3A*.

By exposing developing spinal neurons to a ligand such as *netrin-1* for 30 min, earlier researchers¹² showed that these growth cones attenuate their capacity to turn in response to guidance cues. They also showed that these growth cones recover their sensitivity in a protein synthesis-dependent manner after 60–90 min. Clearly, the time courses of these effects they reported are much longer than the time courses observed in the present experiments. The reasons for this difference are not yet known. It might be because of basic differences in the experimental protocols, such as turning assays versus collapse assays, or the use of attractants by these researchers versus our use of repellents. Furthermore, they used substantially younger spinal neurons that survive in culture on their own yolk supplies¹², whereas we used retinal neurons from older embryos for which L15 medium is essential for survival. Although the time course may be different, it is nevertheless clear that both studies revealed the presence of desensitization followed by resensitization, raising the basic question of the role of these phenomena in growth cone responsiveness.

Desensitization may simply reduce the sensitivity of growth cones to a guidance cue and resensitization may return it to the previous level, as in a simple on-or-off situation. This

'homeostatic reset' mechanism might suggest that directional movements should be intermittent. Indeed, the zigzag tracks of spinal axons that have sometimes been seen in a concentration gradient of a guidance cue have been interpreted as evidence for this¹². Another possibility is that growth cones adapt to the background levels, making them able to respond appropriately to the higher levels of guidance cues riding on top of these background levels. The present study favors such an interpretation, as it shows that the adaptation process allows retinal growth cones exposed to background levels of Sema3A to respond differentially to higher levels of Sema3A than unexposed growth cones. It seems to us that adaptation in a gradient may be an ongoing process comprised of temporally overlapping components, with endocytosis being involved in decreasing the response and translation being involved in increasing the response, and with growth cones using these two mechanisms to adjust their sensitivity to a given background level of ligand.

Although it is easy to understand an adaptation process that allows chemotaxis up a gradient of an attractant, it is more difficult to understand why it might be useful to adapt to a repellent. Yet adaptation to repellents is also seen in bacterial chemotaxis^{26,27}. Our findings are also consistent with another set of earlier findings⁶ showing that exposure of retinal axons to a certain level of ephrins allowed these axons to crawl further up an ephrin gradient. In the retinotectal system, a shallow gradient of repellents covers the anterior-posterior axis of the tectum, and axons have to navigate appropriately within this gradient²⁸. A recent study²⁹ showed that up to a threshold level, ephrinA2 promotes the growth of retinal axons, but above this level it inhibits growth. The threshold level is graded: it is higher for nasal axons than for temporal ones. It is at present unclear how the adaptation process that we have described here relates to these threshold concentrations of ephrinA2 and the endpoints beyond which retinal axons will not grow. This is especially true given that in many animals, retinal axons grow past their most appropriate termination zones, and then form terminal arbors in the correct place by back-branching along the axon shaft while withdrawing their overshooting process^{30,31}.

The first phase of adaptation, desensitization, is dependent on rapid endocytosis. The speed of endocytosis observed here is not uncommon; the somatostatin receptor undergoes internalization and desensitization within 20 s of exposure to somatostatin³², and Sema3A has been reported to elicit a significant increase in fluid-phase endocytosis in axonal growth cones within 5 min¹⁶. The latter report was also the first to correlate Sema3A stimulation of growth cones with endocytosis, and also included a proposed model whereby rearrangement of neuropilin-1 and plexin receptors to F-actin-rich structures within the growth cone was a consequence of Sema3A stimulation¹⁶. The data shown here support this model, with Sema3A stimulating a rapid, endocytosis-dependent depletion of neuropilin-1 from the surface of retinal growth cones. Receptor-mediated endocytosis is preceded by clustering of receptor molecules, followed by internalization through either clathrin- or non-clathrin-mediated pathways¹⁸. Receptor-specific endocytosis as a mechanism of desensitization is consistent with the unchanged sensitivity of growth cones to Sema3A when pretreated with netrin-1, and vice versa. Previous work¹² also showed that *X. laevis* embryonic spinal neurons show homologous, but not heterologous, desensitization. The data presented here strongly support the hypothesis that desensitization occurs at the level of membrane receptors, as the loss of receptors from the growth cone surface via endocytosis is temporally correlated with the desensitization process, and blocking endocytosis prevents desensitization.

Endocytosis of surface receptor may be a cause of desensitization. However, endocytosis may have a much greater role in growth cone navigation. It may be a necessary aspect of receptor signaling. For example, TGF- β receptor internalization to the endosomal compartment has been shown to promote SMAD2 signalling^{18,33}. Furthermore, although

loss of surface receptor can explain desensitization, it is also possible that the receptor remaining on the membrane becomes inactive during desensitization. Indeed, desensitization need not be a receptor-based phenomenon, as components of the signaling pathway downstream of receptor internalization could also be desensitized. The partial replacement of surface receptor when protein synthesis is blocked without a parallel increase in sensitivity would suggest that other components are involved. In this respect members of the MAP kinase (MAPK) family may be of particularly interest. Activation of ERK1/2 is suppressed in some endocytosis-defective cells³⁴, MAPKs such as ERK1/2 have been localized to the endosomal compartment^{35–37} and receptor stimulation can enhance localization of JNK3 to intracellular vesicles³⁸. In growth cones, both Sema3A and netrin-1 signal through various MAPK molecules to induce turning in a gradient or collapse^{12,39}. Thus, it will be necessary to examine the full transduction cascade, from receptor activation to motor response, to find out precisely which components are regulated during the desensitization process.

Our findings may also clarify the role(s) of protein synthesis during growth cone behavior. Our data suggest that resensitization is probably not simply due to the appearance of new receptors on the growth cone surface, as there is a partial replacement of receptor when protein synthesis is blocked, even though there is no recovery of sensitivity in this case. Perhaps, however, the recycled receptors are initially inactive and it is newly synthesized receptors that mediate the resensitization. Because these must represent only a fraction of the total surface receptor to the adapted ligand, a resensitized growth cone would have fewer active receptors than a naive one. This could explain why adapted growth cones show extended sensitivity. If some of the receptors expressed on the surface of resensitized growth cones were newly synthesized, one would expect to find the mRNA for these receptors in the growth cone. However, in preliminary studies, we have been unable to identify either DCC or neuropilin-1 from a retinal growth cone cDNA library (data not shown), which casts some doubt on this model. A growing body of data suggests that growth cones do harbor a subset of mRNA species, including those encoding β -actin^{40,41}, neurofilament⁴², the microtubule-associated protein tau⁴³ and actin-depolymerizing factor⁴⁴. Ligand-induced collapse involves rearrangement of the cytoskeleton, such as a reduction in filamentous actin within the growth cone⁴⁵. The need for protein synthesis during resensitization may reflect production of nascent cytoskeletal elements to be used during collapse and subsequent turning of growth cones. However, such an explanation would not be consistent with a ligand-specific process. Therefore, we suggest that the proteins that are synthesized to mediate resensitization may be involved in replenishing cytosolic components of specific signal transduction pathways. It has previously been demonstrated that protein synthesis is involved in retinal growth cone turning in response to gradients of Sema3A and netrin-1 and growth cone collapse¹⁴. Recently, it was shown, using collapse assays, that rapid protein synthesis is necessary for mouse DRG growth cones to maintain normal sensitivity to Sema3A⁴⁶. These studies indicate that protein synthesis has a role in collapse and turning that may be independent of its role in adaptation. However, as the time course of observable collapse and turning is relatively long compared to the time course of resensitization shown here, interfering with temporal dynamics of growth cone sensitivity could explain all of these effects. Further experiments will be necessary to test this possibility.

METHODS

Retinal cultures

Eye primordia from stage 35/36 *X. laevis* embryos⁴⁷ were cultured in L15 medium without any added growth factors or serum as described previously¹³. Cultures for collapse assays and quantitative immunohistochemistry were grown for 24 h at 20 °C on glass cover slips precoated with 10 μ g/ml poly-L-lysine (Sigma) and 10 μ g/ml laminin (Sigma).

Collapse assays

Collapse assays were performed as described previously^{14,48} with minor modifications. The basal level of collapse ($36.3\% \pm 3.3$ (mean $\pm$ s.e.m.) for Sema3A controls, $n = 1,106$; $34.9\% \pm 2.4$ for netrin-1 controls, $n = 1,056$) was similar to that described previously in cultured *X. laevis* retinal neurons¹⁴. A dose of 1:1 cell supernatant to fresh culture medium (supernatant was from Sema3A-expressing COS-7 cells transiently transfected with Sema3A plasmid, collected after 72 h) gave a collapse rate of $63.0\% \pm 1.3$ ($n = 2,004$), which did not appreciably increase even at higher concentrations of Sema3A. This was defined as the high dose. A 1:4 ratio of supernatant to culture medium consistently gave a collapse rate just above the control value (that is, $43.6\% \pm 2.2$ collapse, $n = 615$), and was defined as the low dose. The intermediate dose of Sema3A used to assess cross-adaptation in Figure 7c was a 1:2 ratio of supernatant to culture medium, which gave a collapse rate of $59.6\% \pm 1.49$ ($n = 745$). For netrin-1 assays, the low dose was 150 ng ml^{-1} ($42.4\% \pm 3.1$ collapse, $n = 921$) and the high dose was 300 ng ml^{-1} ($66.1\% \pm 1.0$ collapse, $n = 961$). Other doses used are described in the legend to Figure 2. The data presented represent at least four independent experiments, with 400 growth cones for each individual data point. Values represent mean percent collapse $\pm$ s.e.m.

Pharmacological reagents

Pharmacological reagents were bath-applied to cultures immediately before the application of the low dose in the collapse or quantitative immunohistochemistry assays, and were $40 \text{ }\mu\text{M}$ anisomycin (Sigma), $25 \text{ }\mu\text{M}$ cycloheximide (Sigma), $50 \text{ }\mu\text{M}$ phenylarsine oxide (Sigma) and $10 \text{ }\mu\text{M}$ monodansyl cadaverine (Sigma). After pretreatment with pharmacological reagents in collapse assays, cultures were washed twice with $900 \text{ }\mu\text{l}$ of culture medium to remove the reagent before exposure to the high dose.

Antibodies and digital quantification of fluorescence intensity

Neuropilin-1 was detected using an anti-neuropilin-1 antibody (Zymed Laboratories Inc.). DCC was detected on growth cones using an anti-DCC antibody (Oncogene Research Products). Growth cones were not permeabilized so as to detect only cell surface receptors. Growth cones were viewed on a Nikon inverted fluorescence microscope with a $100\times$ Plan Apo objective. For quantification, growth cones were randomly selected with phase optics and fluorescent images were captured. The outline of a growth cone was traced digitally and the amount of fluorescence within the area of the growth cone was calculated digitally (Openlab, Improvision; IP Lab, Scanalytics Inc.). The background fluorescence for the same area was similarly calculated and subtracted from the growth cone value. This final value represented the mean fluorescence intensity per unit area. This was repeated to obtain the fluorescence intensities of 30–40 growth cones for each condition per experiment. Values were then normalized against controls. The data presented represent at least four independent experiments. Fluorescent intensities are presented as a percentage of treated growth cones compared to control treated growth cones $\pm$ s.e.m.

Statistical analysis

Statistical analyses were carried out using the Mann-Whitney *U*-test for collapse assays and the Student's *t*-test for the quantitative immunofluorescence assays.

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Figure 1.

Adaptation time course for retinal growth cones. **(a)** To investigate the time course of desensitization and resensitization in retinal growth cones, explant cultures were pretreated with a low dose (LD) of either Sema3A or netrin-1 for 0.5–5 min, before a high dose (HD) of the same molecule was added for 10 min. Cultures were then fixed and the percentage of axons with collapsed growth cones determined. The LD was selected because it caused minimal collapse compared to the basal level of collapse shown by cultures treated with control medium (see Methods). **(b)** Pretreatment with a LD of Sema3A for 2 min or a LD of netrin-1 for 1 min caused a rapid desensitization to a HD, resulting in significantly lower growth cone collapse. After 5 min, growth cones had fully resensitized to a LD of either Sema3A or netrin-1, with collapse rates equivalent to cultures treated with a HD of either cue alone. Asterisks indicate a significant difference ($*P < 0.05$, $**P < 0.005$) between cultures that received both a LD pretreatment and a HD and those that received a HD alone; Mann-Whitney U -test. Error bars, s.e.m.

Figure 2.

Adaptation adjusts sensitivity. Growth cones exposed to a low dose (LD) of Sema3A for 5 min (filled circles), allowing both desensitization and resensitization, were compared to naive growth cones (open squares) on a range of Sema3A doses. The level of Sema3A in the high dose (HD) is normalized to 1.0 and the other doses are expressed as proportions of the normalized HD, with the LD being equivalent to 0.4. Adapted growth cones showed less collapse than naive growth cones at all doses except the HD. The response of naive growth cones saturated at lower doses than the adapted growth cones. At high doses of Sema3A (0.86–1.0), adapted growth cones showed a differential response, but the responses of naive growth cones appeared to saturate at 0.86. Asterisks indicate significant difference (* $P < 0.05$, ** $P < 0.01$), Mann-Whitney U -test. Error bars, s.e.m.

Figure 3.

Resensitization is blocked by protein synthesis inhibitors. **(a,b)** Cultured retinal explants were pretreated with a low dose (LD) of either Sema3A or netrin-1 in the presence of cycloheximide (CHX), an inhibitor of ribosomal translation. Cultures were rinsed after pretreatment to ensure no pharmacological reagents were present during the collapse assay. Inhibition of mRNA translation during pretreatment with a LD of Sema3A **(a)** or a LD of netrin-1 **(b)** prevented resensitization, but not desensitization, to a high dose (HD) of the respective molecule. **(c)** Similarly, pretreatment with anisomycin (Aniso), another protein synthesis inhibitor, and a LD of either Sema3A or netrin-1 for 5 min, prevented resensitization of growth cones. Protein synthesis may be essential to the reacquisition of

responsiveness to guidance cues after desensitization. Asterisks indicate significant difference at $P < 0.05$, Mann-Whitney U -test. Error bars, s.e.m.

Figure 4.

Desensitization is blocked by endocytosis inhibitors. **(a,b)** Cultured retinal explants were pretreated with a low dose (LD) of Sema3A **(a)** or a LD of netrin-1 **(b)** in the presence of PAO, an inhibitor of endocytosis. Cultures were rinsed after pretreatment. Inhibition of endocytosis during pretreatment prevented desensitization of growth cones to a high dose (HD) of the respective molecule, suggesting that endocytosis is necessary for this aspect of adaptation. **(c)** MDC also inhibited desensitization induced by a LD of either netrin-1 (1 min) or Sema3A (2 min) during pretreatment, demonstrating that clathrin-mediated endocytosis may underlie desensitization. Asterisks indicate significant difference (* $P < 0.05$, ** $P < 0.01$), Mann-Whitney U -test. Error bars, s.e.m.

Figure 5.

Neuropilin-1 depletion and replacement in adaptation. (**a–e**) Retinal explant cultures were treated with a low dose (LD) of Sema3A and the amount of neuropilin-1 localized on the surface of the growth cone was assessed by quantitative immunohistochemistry. After 2 min the level of cell surface neuropilin-1 was significantly lower than for the control, but after 5 min it had fully recovered. (**f–k**) Treatment in the presence of cycloheximide (CHX) did not affect the rapid removal of neuropilin-1 from the cell surface after 2 min, but seemed to partially limit its replacement into the membrane after 5 min (**f–h,k**). However, treatment in the presence of PAO abolished the reduction in neuropilin-1 surface staining in response to a LD of Sema3A (**f,i–k**). ** $P < 0.001$ in comparison to controls, Student's t -test. Error bars, s.e.m. Scale bar, 10 μ m.

Figure 6.

DCC depletion and replacement in adaptation. (**a–e**) Retinal explant cultures were treated with a low dose (LD) of netrin-1 and quantitative immunohistochemistry was used to assess the amount of DCC localized on the surface of the growth cone. After 1 min the level of cell surface DCC was significantly lower than the control, whereas after 5 min it had fully recovered. (**f–k**) Treatment in the presence of CHX did not affect the rapid removal of DCC from the cell surface after 1 min; however, inhibition of protein synthesis clearly limited the reinsertion of DCC into the surface membrane (**f–h,k**). PAO abolished the reduction in DCC surface staining in response to a LD of netrin-1 (**f,i–k**). ** $P < 0.001$, * $P < 0.05$ in comparison to controls, Student's t -test. Error bars, s.e.m. Scale bar, 10 μ m.

Figure 7.

Adaptation is ligand specific. **(a)** Cultured retinal explants pretreated with a low dose (LD) of Sema3A for 2 min, washed, then treated with a high dose (HD) of netrin-1 showed a full collapse response. Similarly, cultures pretreated with a LD of netrin-1 (2 min), rinsed, then treated with a HD of Sema3A showed a full collapse response. This suggests that desensitization is ligand specific. **(b,c)** A 5-min pretreatment with a LD of the heterologous cue did not elicit greater sensitivity to the other cue either at a HD **(b)**, nor did a LD of netrin-1 elicit increased sensitivity to a submaximal intermediate dose (ID; 1:2 supernatant/culture medium) of Sema3A **(c)**. Asterisks indicate significant difference (* $P < 0.05$, ** $P < 0.01$), Mann-Whitney U -test. Error bars, s.e.m.