Semaphorin 3A Stimulates Neurite Extension and Regulates Gene Expression in PC12 Cells*§

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The secreted semaphorin 3A (Sema3A) is a member of a large family of proteins that act as guidance signals for axons and dendrites. While the receptors and signaling pathways that mediate the repulsive effects of semaphorins are beginning to be understood in some detail, the mechanisms that are responsible for the ability of Sema3A to stimulate the extension of dendrites remain to be elucidated. Here we show that PC12 cells, a model widely used to study neuronal differentiation, can be used to dissect this pathway. Sema3A is as effective as nerve growth factor in stimulating the extension of neurites from PC12 cells. We show that Sema3A is able to regulate gene expression and identify mitochondria as a novel target of Sema3A signaling. Pharmacological block of mitochondrial reactive oxygen species production abolishes the extension of neurites in response to Sema3A. These results show that the characterization of transcripts that are regulated by axon guidance signals may help to identify novel components of their signaling pathways.

The semaphorins are a large family of secreted and membrane-bound proteins that act as guidance signals for axons and dendrites [reviewed in Ref. 1]. The secreted semaphorin 3A (Sema3A) acts as a chemorepellent for many types of axons including sensory and cortical axons and as an attractant for cortical dendrites (2). The repulsive effects of the secreted class 3 semaphorins are mediated by a receptor complex that contains neuropilin-1 (Nrp-1) or -2 as the ligand binding subunit and an A-type plexin as the signal-transducing subunit (1). Activation of the Sema3A receptor in sensory growth cones results in the depolymerization of actin filaments and the loss of substrate adhesion (1). The molecules implicated in semaphorin signaling include the kinases Fyn, Cdk5, and Fes and members of the Crmp family of microtubule-binding proteins (1). In addition, two types of oxygenases are involved in mediating the repulsive effects of Sema3A, the putative flavoprotein monooxygenases of the MICAL family and lipoxygenase (3, 4).

In addition to its repulsive effects, Sema3A acts as an attractive signal for the apical dendrites of pyramidal neurons (2). In Sema3a knock-out mice, the dendrites of cortical pyramidal neurons are disoriented (2). While mice with a mutation in Nrp-1 that abolishes the binding of semaphorins do not display defects in the orientation of apical dendrites, they show a reduction in the growth of basal dendrites (5). Application of recombinant Sema3A to cortical slice cultures increases the extension and branching mainly of basal dendrites (6). The effects of Sema3A depend on the intracellular cGMP concentration in the target cells (7). Elevation of cytosolic cGMP converts the repulsion of axons by Sema3A into an attraction and blocks the collapse of sensory growth cones, suggesting that these pathways have at least some components in common. However, with the exception of cGMP, the factors responsible for mediating the attractive or stimulatory effects of semaphorins remain to be elucidated.

Sema3A and Sema3E are both able to stimulate the mitogen-activated protein kinase (MAPK) pathway, while Sema3F antagonizes the activation of p38 activation by NGF (8–10). In Xenopus retinal ganglion cell growth cones, local translation and p42/p44 MAPK activation are required for the response to Sema3A (11). MAPKs are central elements in signaling pathways that regulate gene expression in response to extracellular signals. The ability to activate MAPKs suggests that semaphorins may also be able to modulate gene expression. Here we show that in PC12 cells, a cell line that is widely used as a model for neuronal differentiations (12), Sema3A induces the extension of neurites and regulates gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture—PC12 cells were cultured on fibronectin (Sigma) in RPMI 1640 medium supplemented with 100 units/ml penicillin, 10% fetal calf serum (FCS), and 5% horse serum (Invitrogen). 293T cells were grown in minimal essential medium supplemented with 100 units/ml penicillin and 10% FCS (Invitrogen). AP-Flag-Sema3A was produced by a stably transfected cell line (cell line 293–717; Ref. 13) and added to cultures of PC12 cells or hippocampal neurons as conditioned medium. Conditioned medium was obtained by incubating the cells for 48 h with serum-free medium and supplemented subsequently with 1.5% FCS. Medium conditioned by 293T cells was used as a control. The induction of neurites was quantified by staining the cells with rhodamine-phalloidin and determining the total length of neurites per cell using the UTHSCSA ImageTool v3.00 software.

Binding Assay—2 × 10⁴ PC12 cells were seeded onto fibronectin.
conted coverslips in a 24-well plate. After 24 h of culture the cells were incubated with Sema3A or control medium for 1 h, fixed with 4% formaldehyde in phosphate-buffered saline, and stained with the anti-FLAG M2 antibody (1:200 dilution; Sigma) to detect Sema3A and rhodamine-phalloidin (1:350 dilution; Molecular Probes). The goat Alexa Fluor 488 anti-mouse antibody (Molecular Probes) was used at a dilution of 1:1000.

Semi-quantitative PCR—RNA was isolated from PC12 cells using thepeqGOLD kit (peqLab) according to manufacturer's instructions. Semi-quantitative PCR was performed with gene specific primers (supplemental Table 1) as described (14). Samples were taken after 25, 30, and 35 cycles, separated by electrophoresis, and stained with ethidium bromide. The ratio of induction or repression was calculated from the fluorescence intensity values, which were determined using the BioDe- cAnalyze software (Biometra). The blocking anti-Nrp-1 antibody was directly added to the conditioned medium at a concentration of 1.25 μg/ml (15). Hippocampal neurons from E18 rat embryos were cultured as described before (16).

Western Blot Analysis—PC12 cells were lysed in 2% Triton X-100 in phosphate-buffered saline and solubilized by incubation at 4 °C for 30 min. The samples were separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schull), and Western blots were developed using the Vectastain ABC kit (Vector Laboratories) according to manufacturer's instructions. The following primary antibodies were used: anti-Sema5A (1:2000), anti-Crmp2 (1:1000), and anti-Crmp4 (1:1000) (17). The anti-Sema5A antibody was raised against the C-terminal 130 amino acids expressed in bacteria using the expression vector pQE-30 (Qiagen). Recombinant protein was purified by affinity chromatography on nickel-nitrilotriacetic acid-agarose (Qiagen) and used as antigen to immunize rabbits (Eurogentech). Biotinylated anti-rabbit and horseradish peroxidase-coupled anti-mouse antibodies (Mo-BiTec) were used at a dilution of 1:19,000. To analyze the activation of the MAPK pathway cell lysates were incubated overnight at 4 °C with immobilized anti-phospho-p42/p44 antibody (Cell Signaling Technology). Bound proteins were eluted with SDS sample buffer and analyzed by Western blot using the anti-phosphotyrosine antibody P-Tyr-100 (Cell Signaling Technology). Loading of comparable amounts of protein was confirmed with an anti-α-tubulin antibody (clone 6-112-1; Sigma).

RESULTS

Sema3A Induces the Extension of Neurites by PC12 Cells—
Assays with recombinant Sema3A confirmed the presence of Sema3A-binding sites on PC12 cells (data not shown). Incubation with Sema3A for 12 h induced the extension of neurites to a similar extent as NGF (Fig. 1, A and B). The formation of neurites was seen only after prolonged treatment with Sema3A for 12 h but not when Sema3A was removed after 1 h of culture and replaced by normal medium (data not shown). Neurite extension in response to Sema3A but not to NGF was completely abolished by a blocking anti-Nrp-1 antibody (Fig. 1, A and B). To exclude an indirect effect of Sema3A on neurite extension by increasing the sensitivity of PC12 cells for the low amount of endogeneously produced NGF as shown for Sema4D (18), we treated PC12 cells with Sema3A or NGF and an inhibitor of the NGF receptor TrkA (K-252a). The stimulation of neurite extension by NGF was completely blocked by K-252a, while the length of neurites induced by Sema3A was not affected (Fig. 1B).

Activation of the MAPK pathway by NGF is crucial for the differentiation of PC12 cells (12). In Western blots an anti-phosphoryosine antibody detected a transient activation of p42/p44 MAPK in PC12 cells that was maximal at 15 min after addition of Sema3A or NGF (Fig. 1C). No activation was detected in cells treated with control medium without Sema3A. This effect was selective for p42/p44 MAPK as the phosphorylation of p38 was not changed by Sema3A (data not shown). The MAPK inhibitor PD98059 (19) completely blocked the stimulation of neurite extension by NGF and Sema3A showing that activation of MAPK is essential for the effects of Sema3A (Fig. 1B).

Sema3A Regulates Gene Expression in PC12 Cells and Neurons—The activation of the MAPK pathway suggested that Sema3A may induce neurites not only by its effects on the cytoskeleton but also by regulating transcription. To address this question in the absence of obvious target genes for Sema3A, we monitored the response to Sema3A by using cDNA microarrays. PC12 cells were incubated for 12 h with Sema3A or control medium, the RNA was isolated, and reverse-transcribed cDNA was hybridized to two cDNA microarrays with largely non-overlapping sets of 7500 human (array 1) and 3500 mouse genes of known function (array 2) (20). Of the 2681 cDNAs, 1722 genes (array 1) and 2306 genes (array 2) expressed in PC12 cells, Sema3A regulated the expression of 130 (array 1: 97 up-regulated and 49 down-regulated) and 146 genes (array 2: 50 up-regulated and 80 down-regulated), respectively (supplemental Tables 2–5). Interestingly, several genes coding for factors known to be involved in mediating or modulating the effects of semaphorin signaling were induced 2–5-fold by Sema3A (supplemental Table 5). These included Plxna1 (encoding Plexin-A1), CRMp4 (Crmp4), and GUCY1A3 (guanylate cyclase). The RNA levels for other components of the Sema3A signaling pathway like the ligand-binding subunit Nrp-1 were unchanged. On the other hand, the expression of several transcripts for mitochondrial proteins such as different NADH dehydrogenase subunits (NDUFB3, NDUF28) was repressed between 2- and 12-fold (supplemental Table 5). However, not all mRNAs for mitochondrial proteins were affected. Only two (NDUFB3, NDUF28) out of 15 transcripts coding for different subunits of NADH dehydrogenase (array 1: 12 cDNAs, array 2: 3 cDNAs) and expressed in PC12 cells were regulated by Sema3A.

Based on the results of the microarray experiments, the regulation of selected genes by Sema3A and NGF was analyzed by semiquantitative PCR. These also included transcripts, like Crmp2 or Sema5a, that were not present on the microarrays. The semiquantitative PCR confirmed that expression of Plxna1, Crmp2, Crmp4, and Gucy1A3 was induced 2–8-fold by Sema3A while that of Sema5a and Ndufb3 was decreased. Although the extent of induction or repression differed from the microarray hybridization data, the regulation of all transcripts

![Fig. 1. Sema3A induces the extension of neurites by PC12 cells.](http://www.jbc.org/Downloaded from http://www.jbc.org by guest on July 25, 2018)
Sema3A Stimulates Neurite Extension

Table I
Sema 3A regulates gene expression in PC12 cells and hippocampal neurons

| Transcript (protein) | PC12 + Sema3A | PC12 + Sema3A + α-Nrp-1 | PC12 + NGF | Neurons + Sema3A |
|----------------------|---------------|-------------------------|------------|------------------|
| Actb (β-actin) | 1.1 (± 0.3) | 1.0 (± 0.1) | 1.2 (± 0.2) | 1.0 (± 0.3) |
| Vegfa (VEGF-A) | 2.0 (± 0.2) | 1.1 (± 0.1) | 2.5 (± 0.4) | 1.7 (± 0.6) |
| Crmp2 (Crmp2) | 8.0 (± 1.1) | 1.2 (± 0.3) | 9.6 (± 1.2) | 9.0 (± 3.1) |
| Crmp4 (Crmp4) | 2.0 (± 0.6) | 0.9 (± 0.1) | 4.3 (± 0.8) | 5.0 (± 1.8) |
| Plexna1 (Plexn-A1) | 5.0 (± 1.6) | 1.3 (± 0.1) | 1.1 (± 2.1) | 5.9 (± 2.8) |
| Gucy1a3 (guanylate cyclase 1) | 1.7 (± 0.3) | 1.1 (± 0.2) | 1.0 (± 0.2) | 1.7 (± 0.5) |
| Cacna1c (L-type calcium channel) | −4 (± 0.5) | 1.2 (± 0.3) | −3.1 (± 0.6) | −3.2 (± 1.3) |
| Sema5a (Sema 5A) | −2 (± 0.4) | 1.3 (± 0.3) | 1.1 (± 0.1) | NA |
| Nck1 (Nck) | −1.6 (± 0.3) | 0.8 (± 0.1) | −3.2 (± 0.9) | −1.4 (± 0.8) |
| Ndub3 (NADH dehydrogenase) | −3.3 (± 1.2) | 1.2 (± 0.3) | −1.1 (± 0.3) | −2.3 (± 0.9) |
| Pdhx (pyruvate dehydrogenase) | −5.5 (± 0.9) | −0.8 (± 0.2) | 1.2 (± 0.4) | −2.6 (± 0.7) |

PC12 cells or hippocampal neurons were cultured for 12 h in medium containing Sema3A, NGF, or control medium. RNA was isolated and used for semi-quantitative reverse transcriptase-PCR. The ratios of the mRNA amounts in treated and control cells are listed. The value for Actb was 1.1 (the amount of RNA was identical in both samples), and the transcript for β-actin was used as a control. Addition of a blocking anti-Nrp-1 antibody (+α-Nrp-1) abrogated the effects of Sema3A on gene expression. NA, not analyzed.

Fig. 2. Sema3A regulates expression of CRMP2, CRMP4, and Sema5A. A, PC12 cells were cultured for 12 h in medium containing Sema3A (+Sema3A) or control medium (−Sema3A). RNA was isolated after 12 h and used for semiquantitative PCR for the indicated genes. The PCR products after 25 cycles are shown. The mRNA for β-actin was used as a control and showed no changes. These results confirm that the regulation of gene expression by Sema3A can be detected both at the RNA and the protein level. B, Incubation with the radical scavenger N-acetyl-l-cysteine blocked the extension of neurites in response to Sema3A but not to NGF (Fig. 3).

Discussion
In addition to its repulsive effects, Sema3A has the ability to stimulate the extension of dendrites by cortical neurons (2, 6). Here we show that PC12 cells can serve as a model system to dissect the pathways that mediate the stimulatory effect of Sema3A. Sema3A is as potent as NGF in the induction of neurite extension by PC12. Our results demonstrate that Sema3A has long term effects by regulating gene expression and identify some of its targets. Gene expression in PC12 cells is probably regulated through MAPKs, as Sema3A selectively activated the p42/p44 MAPK pathways, respectively, is required for their effects on growth cones (11, 25, 26). Stimulation of translation by Netrin-1 and Sema3A through the p38 and p42/p44 MAPK pathways, respectively, is required for their effects on growth cones (11, 25, 26). Stimulation of translation via the MAPK pathway is required for the resensitization of axons that lose their responsiveness over time in a netrin-1 gradient (26). A similar feedback pathway may be involved in the response to Sema3A and include the increased expression of the Sema3A-responsive transcripts.

To investigate whether Sema3A is able to regulate gene expression not only in a cell line but also in neurons, cultures of hippocampal neurons isolated from newborn rat embryos were incubated for 4 days in vitro with Sema3A for 12 h. A comparison of expression levels to neurons cultured in control medium by semiquantitative PCR confirmed that all analyzed genes were regulated as described for PC12 cells and differed only in the ratios of induction or repression (Fig. 2A; Table I).

Mitochondrial ROS Production Is Required for Sema3A-induced Neurite Extension—Interestingly, Sema3A reduced the levels of 17 transcripts encoding mitochondrial proteins (Table I and supplemental Tables 2–5). The repression of one of these, Ndub3, was also confirmed in hippocampal neurons. Mitochondria are the most important source of ROS production (21). ROS are involved in different signaling pathways and can be accompanied by changes in mitochondrial metabolism (22, 23). The regulation of several transcripts encoding mitochondrial proteins suggested an involvement of ROS production in the response to Sema3A. To test this possibility, PC12 cells were incubated with Sema3A or NGF and the production of ROS inhibited by rotenone or 1-methyl-4-phenylpyridinium. For these experiments, a concentration of the inhibitors was chosen that did not induce apoptosis (supplemental Fig. S1) (24). Pharmacological inhibition of ROS production completely blocked the extension of neurites in response to Sema3A, while it did not interfere with neurite induction by NGF (Fig. 3, A and B). Incubation with the radical scavenger N-acetyl-l-cysteine
Scavenger N by Sema3A but not by NGF. Way changes the balance of Rho and Rac activity leading to the collapse of growth cones (1). Rho and Rac also regulate the extension and retraction of neurites (29). Active Rac promotes extension, while Rho activation induces retraction. Rac down-regulates Rho through the stimulation of ROS production (22). ROS inactivate the low molecular weight protein-tyrosine phosphatase by oxidizing its catalytic residue leading to an increase in tyrosine-phosphorylation and activation of p190RhoA (GAP, GTPase-activating protein) (30, 31). Stimulation of mitochondrial ROS production by Sema3A might inhibit Rho activity, which would favor neurite extension.

In summary, the identification of Sema3A target genes not only suggests a role for the regulation of gene expression in the response to axon guidance signals but also allows the identification of possible new components of the signaling pathways mediating their effects. This approach allowed us to reveal mitochondrial ROS production as a component of the Sema3A signaling pathway.

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of Sema3A receptor subunits and other components of the signaling pathway. Sema3A stimulates endocytosis and the redistribution of the Sema3A receptor to vesicles in sensory growth cones (27). The endocytosis of membrane-bound proteins is the first step in the degradation of activated receptors. An increased production of Plexin-A1 and Crmps could be a mechanisms to replenish degraded receptor subunits and components of the signaling cascade to sustain the responsiveness of cells to Sema3A. The intracellular concentration of cyclic nucleotides determines the response to guidance signals (7). High cGMP concentrations convert the repulsion of axons by Sema3A to an attraction. An increased production of guanylate cyclase would, therefore, also promote the extension of neurites.

The ability of cGMP to determine the direction of the response to Sema3A suggests that the pathways mediating repulsion and attraction have at least some components in common. Here we identify a novel target of Sema3A and show that ROS production by mitochondria is required for Sema3A-dependent neurite extension. Sema3A may directly regulate genes encoding mitochondrial proteins through MAPKs as shown for cytokines (28). Alternatively, the down-regulation may represent an indirect effect of Sema3A and reflect a response to the increase in ROS production that can be accompanied by changes in mitochondrial metabolism (23).

The effects of Sema3A on the cytoskeleton are mediated in part by small GTPases (1, 29). Activation of the Sema3A pathway changes the balance of Rho and Rac activity leading to the collapse of growth cones (1). Rho and Rac also regulate the extension and retraction of neurites (29). Active Rac promotes extension, while Rho activation induces retraction. Rac down-regulates Rho through the stimulation of ROS production (22). ROS inactivate the low molecular weight protein-tyrosine phosphatase by oxidizing its catalytic residue leading to an increase in tyrosine-phosphorylation and activation of p190ROA (GAP, GTPase-activating protein) (30, 31). Stimulation of mitochondrial ROS production by Sema3A might inhibit Rho activity, which would favor neurite extension.

In summary, the identification of Sema3A target genes not only suggests a role for the regulation of gene expression in the response to axon guidance signals but also allows the identification of possible new components of the signaling pathways mediating their effects. This approach allowed us to reveal mitochondrial ROS production as a component of the Sema3A signaling pathway.
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