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ORIGINAL ARTICLE

A retrograde apoptotic signal originating in NGF-deprived distal axons of rat sympathetic neurons in compartmented cultures

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Previous investigations of retrograde survival signaling by nerve growth factor (NGF) and other neurotrophins have supported diverse mechanisms, but all proposed mechanisms have in common the generation of survival signals retrogradely transmitted to the neuronal cell bodies. We report the finding of a retrograde apoptotic signal in axons that is suppressed by local NGF signaling. NGF withdrawal from distal axons alone was sufficient to activate the pro-apoptotic transcription factor, c-jun, in the cell bodies. Providing NGF directly to cell bodies, thereby restoring a source of NGF-induced survival signals, could not prevent c-jun activation caused by NGF withdrawal from the distal axons. This is evidence that c-jun is not activated due to loss of survival signals at the cell bodies. Moreover, blocking axonal transport with colchicine inhibited c-jun activation caused by NGF deprivation suggesting that a retrogradely transported pro-apoptotic signal, rather than loss of a retrogradely transported survival signal, caused c-jun activation. Additional experiments showed that activation of c-jun, pro-caspase-3 cleavage, and apoptosis were blocked by the protein kinase C inhibitors, rottlerin and chelerythrine, only when applied to distal axons suggesting that they block the axon-specific pro-apoptotic signal. The rottlerin-sensitive mechanism was found to regulate glycogen synthase kinase 3 (GSK3) activity. The effect of siRNA knockdown, and pharmacological inhibition of GSK3 suggests that GSK3 is required for apoptosis caused by NGF deprivation and may function as a retrograde carrier of the axon apoptotic signal. The existence of a retrograde death signaling system in axons that is suppressed by neurotrophins has broad implications for neurodevelopment and for discovering treatments for neurodegenerative diseases and neurotrauma.

Keywords: nerve growth factor, apoptosis, retrograde apoptotic signal, sympathetic neuron, axon, glycogen synthase kinase 3, c-jun

Introduction

During development, neurotrophin-responsive neurons extend their axons to innervate target cells releasing neurotrophins. Neurotrophins bind to cognate receptors on axon terminals activating mechanisms supporting neuronal survival and differentiation. It is generally believed that target cells regulate their innervation by releasing neurotrophins in limiting amounts during development, and only neurons obtaining sufficient neurotrophin signals survive [1]. This process is a major contributor to the programmed cell death that eliminates about 50% of neurons during normal development [2].

The prototypical neurotrophin, nerve growth factor (NGF), elicits many of its biological effects through activation of the TrkA receptor [3]. It has been known for over 30 years that NGF applied to sympathetic axon terminals *in vivo* is retrogradely transported along the axons to the cell bodies [4], suggesting that NGF bound to its

receptor and internalized at the nerve terminal is transported to the cell bodies where it generates its survival effect [5]. Subsequent studies providing evidence for retrograde transport of phosphorylated TrkA [6] and the co-occurrence of NGF and TrkA in endosomal preparations [7] have supported this view, now known as the "signaling endosome hypothesis" [8, 9].

However, evidence from our laboratory indicates that signals initiated by NGF applied to distal axons of rat sympathetic neurons in compartmented cultures support neuronal survival without transport of NGF to the cell bodies [10] and with no requirement for the appearance of phosphorylated TrkA in the cell bodies [11, 12]. These data suggest that phosphorylated TrkA in distal axons

activates downstream signaling mechanisms in the distal axons that communicate to the cell bodies. Here, we present evidence for a fundamentally different retrograde survival mechanism in which NGF at distal axons suppresses an apoptotic signaling mechanism in distal axons. This axon apoptotic signal, when derepressed in response to NGF withdrawal, sends a pro-apoptotic signal retrogradely to the cell body to promote the apoptotic program.

Results

For these investigations neurons were plated in compartmented cultures and maintained for 5-7 days with 10
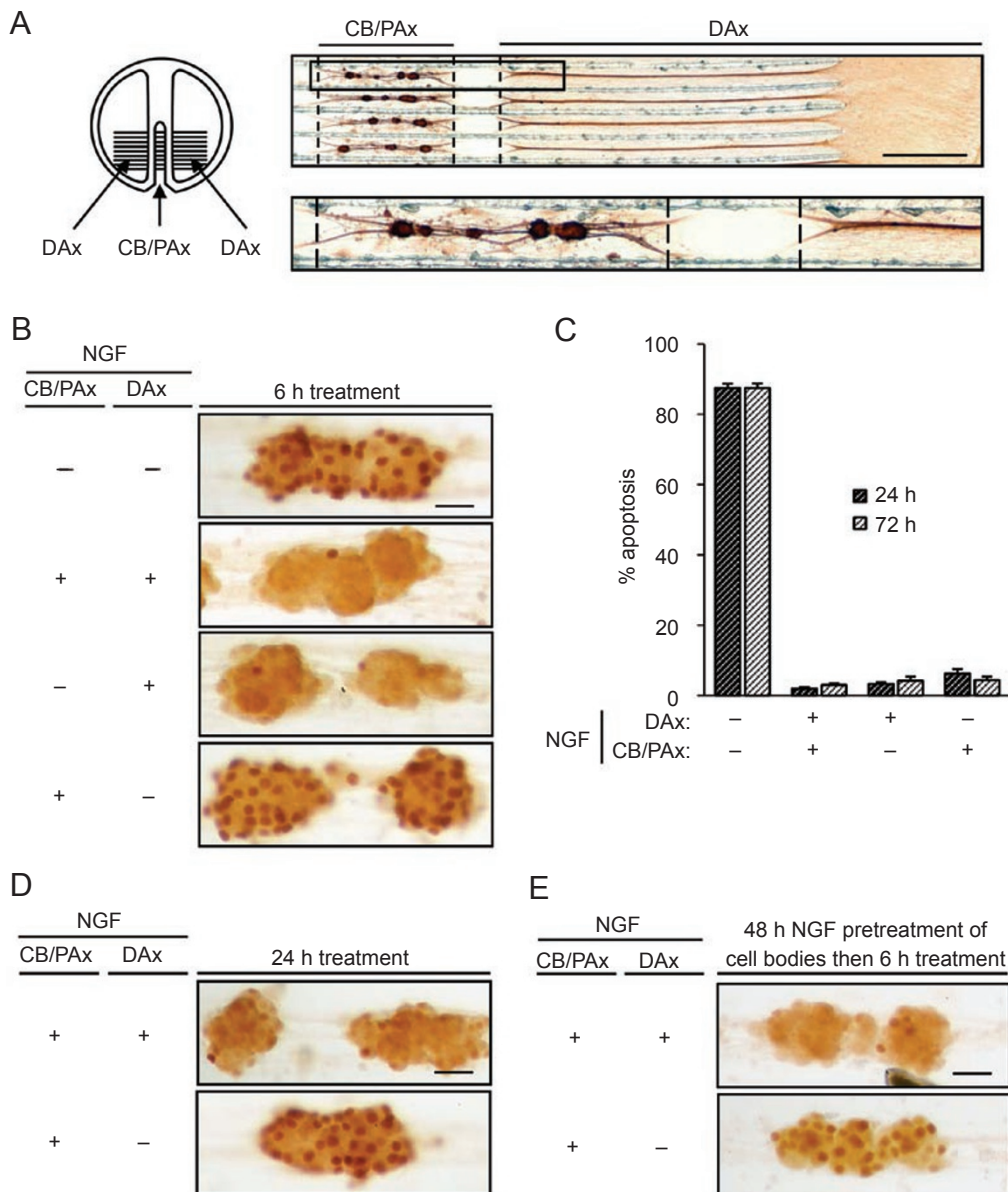
ng/ml NGF supplied to the cell bodies and proximal axons (CB/PAx) and 50 ng/ml NGF supplied to distal axons (DAx) before initiating the experimental treatments (Figure 1A). To assess the effects of NGF in our experiments, CB/PAx and/or DAx were given either media containing 50 ng/ml NGF to maintain robust NGF signaling or media lacking NGF and instead supplied with 24 nM NGF antibodies to terminate all NGF signaling. Activation of the apoptotic program in the cell bodies was assessed after 6 h of experimental treatment by monitoring the phosphorylation (Ser 63) of the pro-apoptotic transcription factor, c-jun, by immunocytochemistry or immunoblot. c-jun is activated early during the apoptosis of NGF-deprived sympathetic neurons [13] by phosphorylation of Ser 63 and 73 residues by JNKs [14, 15]. NGF withdrawal induces phosphorylation of c-jun and its translocation to the nucleus [16] where it promotes expression of pro-apoptotic proteins such as BIM [17]. Deletion of c-jun in sympathetic neurons prevents apoptosis in response to NGF withdrawal [18] indicating a requirement of c-jun for NGF deprivation-induced apoptosis. Apoptosis was assayed by Hoechst staining of nuclei; an apoptosed neuron contained a condensed nucleus after 24 h and no nucleus after 48 or 72 h of treatment.

Local withdrawal of NGF from distal axons induces an apoptotic signal that activates c-jun in cell bodies

To assess how NGF supplied to DAx *versus* cell bodies affects the state of c-jun activation, CB/PAx and/or DAx were supplied with NGF or deprived of NGF for 6 h and then assayed for nuclear accumulation of activated c-jun. NGF-deprived neurons displayed nuclear staining, but neurons supplied with NGF either in all compartments or only at their DAx, both conditions that support survival (see Figure 1C), displayed virtually no nuclear staining (Figure 1B). Unexpectedly, when NGF was supplied directly to CB/PAx but removed from DAx, nuclear accumulation of phosphorylated c-jun was observed, similar to cultures completely deprived of NGF (Figure 1B), but the neurons did not undergo apoptosis for at least 72 h, the longest time observed (Figure 1C). Nuclear staining persisted for at least 24 h in neurons with NGF-deprived DAx (Figure 1D). Neurons pretreated for 48 h with 50 ng/ml NGF in all compartments, to ensure that survival signaling mechanisms in the cell bodies were fully stimulated, still displayed nuclear accumulation of phosphorylated c-jun in response to withdrawal of NGF from their DAx (Figure 1E). In light of these results, we hypothesize that the loss of NGF signaling in DAx generates an apoptotic signal in the axons that travels retrograde to the cell bodies to produce c-jun activation. We further hypothesize that survival mechanisms activated by NGF supplied to the CB/PAx cannot deactivate the retrograde apoptotic signal that activates c-jun, and instead support survival by acting downstream of c-jun to counteract the apoptotic signal arriving from DAx.

The axonal apoptotic signal requires microtubule-based transport in axons

NGF is retrogradely transported [19] along microtubules in a dynein-dependent process [20] and was therefore used as a marker of microtubule-based retrograde

Figure 1 Sustained activation of the pro-apoptotic molecule, c-jun, is induced by local NGF withdrawal at the DAx even when NGF survival signals are supplied directly to the cell bodies. **(A)** Schematic diagram of a compartmented culture and images of sympathetic neurons in a 1-week-old culture immunostained for tubulin. The schematic diagram (left) shows a Teflon divider that partitions a 35-mm tissue culture dish (dish not shown) into compartments that separate the media supplied to the cell bodies/proximal axons (CB/PAx) from media supplied to the distal axons (DAx) of sympathetic neurons cultured from newborn rats. The divider is applied to the dish using a thin layer of silicone grease. Dissociated neurons are plated in the center compartment, and axons extend left and right under the barriers and into the distal compartments. The upper right panel shows the CB/PAx in the center compartment and DAx in the right compartment in four tracks, out of a total of 16, in a compartmented culture. The scale bar equals 1 mm. The lower right panel is a higher magnification of the part of a single track boxed in the top panel. Dotted lines indicate barrier boundaries. Axons under the barriers are unstained. **(B)** c-jun is activated when NGF is removed from DAx. Compartments were supplied with 50 ng/ml NGF (+) or media lacking NGF and containing NGF antibodies (−) as indicated for 6 h. Following treatment, CB/PAx were processed for immunocytochemistry with an antibody recognizing phosphorylated c-jun (Ser 63). c-jun activation is characterized by nuclear accumulation of phosphorylated c-jun. The scale bar equals 50 μm. Results are representative of three independent experiments. **(C)** Local withdrawal of NGF from DAx does not result in apoptosis if NGF is provided to the CB/PAx. NGF (+) or NGF antibodies (−) were supplied as indicated for 24 or 72 h. Quantification of apoptosis is the percentage of neurons with condensed nuclei per group. At 72 h, neurons lacking nuclear staining were also included in the count (see Materials and Methods). A minimum of 250 neurons were assessed per culture. Data were compiled from two experiments (± SEM, *n*=6 cultures). **(D, E)** Cultures were treated and analyzed as in (B). Results are representative of three independent experiments. The scale bar equals 50 μm. (D) c-jun activation following 24-h treatment. (E) CB/PAx were pretreated for 48 h with 50 ng/ml NGF in all compartments prior to experimental treatment.

transport. The retrograde transport of Cy3-labeled NGF applied to DAx was abolished by the application of the microtubule-destabilizing drug, colchicine (10 μM), to DAx (Figure 2A). Colchicine applied to DAx of NGF-deprived neurons substantially inhibited the activation of c-jun in CB/PAx (Figure 2B) suggesting that c-jun is activated by a retrograde death signal from DAx rather than by the loss of a retrograde survival signal from DAx. Colchicine application to DAx in the presence of NGF, on its own, produced a low level of c-jun activation simi-

lar to that observed in colchicine-treated, NGF-deprived neurons suggesting that this slight activation may be a side effect of colchicine.

We attempted to determine if blocking axonal transport would prevent apoptosis during NGF deprivation. Unfortunately, colchicine application resulted in the degradation of DAx starting at 12 h (data not shown). Therefore, experiments measuring neuronal survival after 24 h of colchicine treatment were not feasible.

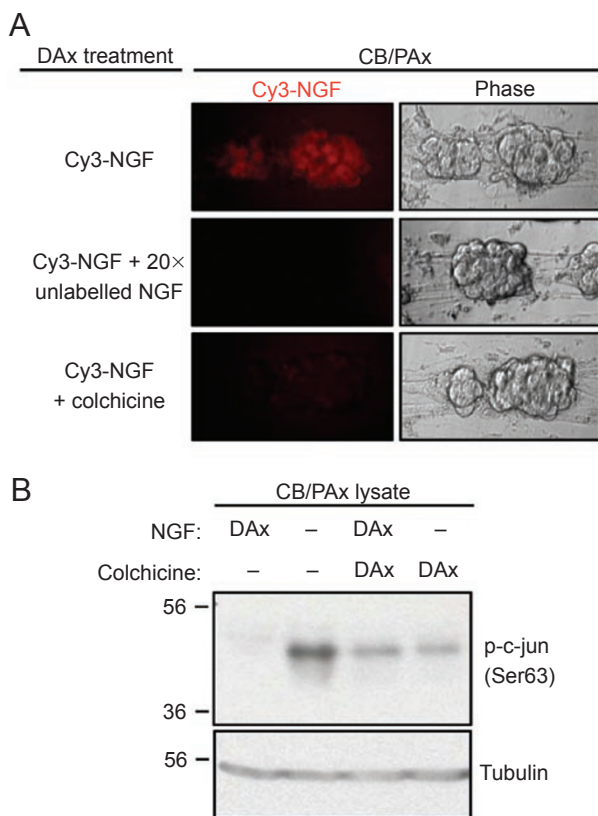*Rottlerin and chelerythrine block the retrograde apoptotic signal only when applied to distal axons*

While screening a variety of compounds (data not shown), we found that rottlerin (2.5 μM) almost completely prevented nuclear accumulation of phosphorylated c-jun only when applied to DAx (Figure 3A and 3B). Rottlerin did not itself cause c-jun activation when applied either at the DAx or the CB/PAx of cultures maintained with NGF in all compartments.

Rottlerin was originally characterized as a protein kinase C (PKC) inhibitor with specificity for the novel PKC isoform delta (PKCδ, IC50=3-6 μM in rat brain) over other PKC isoforms [21]. We compared the effects of rottlerin with the effects of two other PKC inhibitors with specificities for different PKC isoforms: chelerythrine, a general PKC inhibitor [22] and Gö6976, an inhibitor of conventional PKC isoforms but not novel PKC isoforms such as PKCδ [23]. The indicated treatments (Figure 4A) were applied to NGF-deprived neurons for 24 h after which neurons were assayed for apoptosis.

NGF-deprived neurons (black bars) without drug treatment underwent 84% apoptosis, but only 32% and 48% with rottlerin (2.5 μM) or chelerythrine (10 μM) respectively supplied to DAx. Neither drugs prevented apoptosis when applied to CB/PAx. Chelerythrine applied to DAx, like rottlerin, prevented c-jun activation in NGF-deprived neurons (Supplementary information, Figure S1). Rottlerin's protection lasted for at least 72 h (Figure 4B). Gö6976 was not protective from either site of application (Figure 4A). Since PKCδ is a target of both rottlerin and chelerythrine, but not Gö6976, these results initially suggested that PKCδ might be required for generating the apoptotic signal in axons, but see below.

Figure 2 Colchicine applied to distal axons (DAx) during NGF withdrawal blocks activation of c-jun in the cell bodies. **(A)** Colchicine blocks retrograde transport of Cy3-labeled NGF. Cy3-labeled NGF (50 ng/ml) was applied to DAx for 6 h. Colchicine (10 μM) or 20-fold excess unlabeled NGF (1 μg/ml) was also supplied to DAx during treatment as indicated. Cell bodies/proximal axons (CB/PAx) were monitored for the accumulation of Cy3-labeled NGF. Results are representative of three independent experiments. **(B)** Colchicine applied to DAx blocks c-jun activation during NGF withdrawal. DAx were supplied with 50 ng/ml NGF or NGF antibodies (−) in the absence or presence of 10 μM colchicine where indicated. CB/PAx in all cultures were treated with NGF antibodies. Following a 6 h treatment, CB/PAx lysates were analyzed by immunoblotting with an antibody recognizing phosphorylated c-jun (Ser 63). The blot was reprobed for β-tubulin as a loading control. Results are representative of four independent experiments.
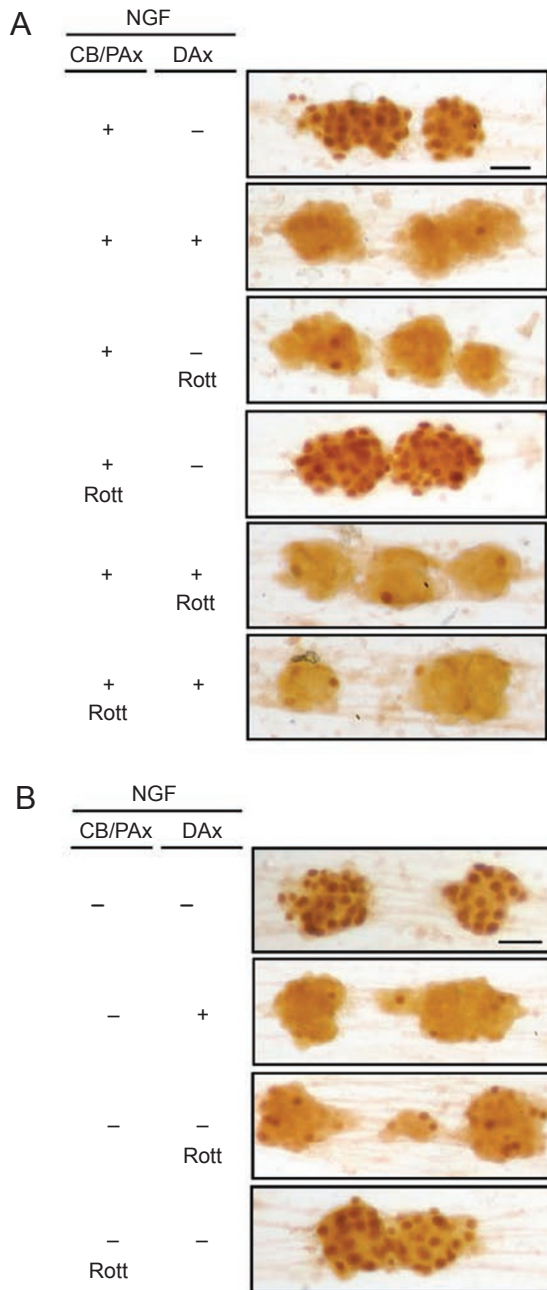
The cleavage of pro-caspase-3 to caspase-3 in the cell bodies/proximal axons is blocked by rottlerin only when applied to distal axons

Cleavage of pro-caspase-3 to its active form, caspase-3, is a required step occurring late in sympathetic neuron apoptosis [24]. To determine if pro-caspase-3 cleavage requires the apoptotic signal from DAx, sympa-

Figure 3 Rottlerin acts exclusively on distal axons (DAx) to prevent activation of pro-apoptotic c-jun. Compartmented cultures were supplied with 50 ng/ml NGF (+) or media lacking NGF and containing NGF antibodies (−) in the presence or absence of rottlerin (Rott, 2.5 μM) to the cell bodies/proximal axons (CB/PAx) or the DAx as indicated for 6 h. Then cultures were analyzed as in Figure 1B. The scale bar equals 50 μm. **(A)** Rottlerin when applied to DAx, but not when applied to CB/PAx, prevented c-jun activation caused by local withdrawal of NGF from DAx. Results are representative of three independent experiments. **(B)** Rottlerin when applied to DAx, but not when applied to CB/PAx, prevented c-jun activation caused by complete withdrawal of NGF. Results are representative of four independent experiments.

thetic neurons were deprived of NGF for 21 h with different distributions of rottlerin. Immunoblot analysis of lysates showed a 17 kDa band corresponding to cleaved pro-caspase-3 appearing in CB/PAx, but not in DAx of NGF-deprived neurons (Figure 4C). Examination of CB/PAx lysates showed that NGF deprivation greatly increased the intensity of the caspase-3 band compared with control cultures maintained with NGF at their DAx (Figure 4C and 4D). Rottlerin prevented the increase in caspase-3 only when applied to DAx (Figure 4D).

Rottlerin inhibits the axonal apoptotic signal at axon terminal segments
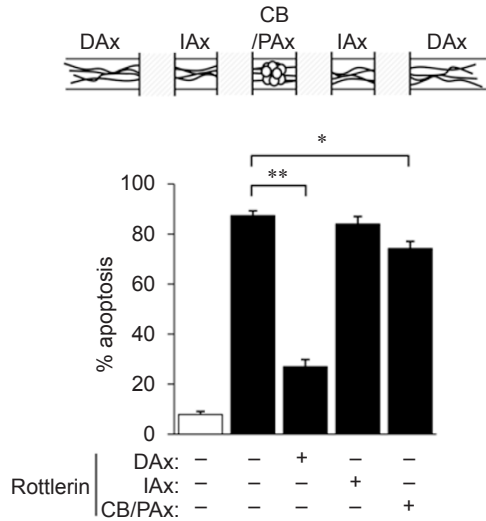
Sympathetic neurons were grown in five-compartmented cultures in which the neurons extend their axons across one barrier, through a 1-mm-wide intermediate compartment, and then across a second barrier into the distal compartment, extending a distance of greater than 2 mm. After 5 days in culture, the intermediate axons (IAx) and CB/PAx were deprived of NGF for 2 days to eliminate neurons with axons that had not crossed into the distal axon compartment. Neurons were then treated with NGF on DAx or completely deprived of NGF in the presence or absence of rottlerin in the indicated compartments for 24 h and assessed for apoptosis (Figure 5). NGF deprivation caused 87% of neurons to undergo apoptosis. Apoptosis was reduced to 27% when rottlerin was supplied to DAx, but rottlerin applied to IAx did not provide significant protection (84% apoptosis). Rottlerin applied to CB/PAx offered a small but significant protection (74% apoptosis). These results indicate that the axonal apoptotic signal blocked by rottlerin mainly originates in the distal axon segments and is retrogradely transported by downstream signaling molecules through the IAx to the cell bodies. This suggests that the axon terminals may be the site of origin of the retrograde apoptotic signal.

*The axonal apoptotic signal is not sensitive to PKCδ knockdown by siRNA*

PKCδ has been implicated in mediating glutamate toxicity in primary cortical neurons [25] and mediating the effects of neurotoxins and oxidative stress in dopaminergic neurons [26]. Since rottlerin and chelerythrine both inhibit PKCδ, we investigated if PKCδ activity was required to generate the retrograde apoptotic signal in DAx. To knock down the expression of PKCδ, neurons were transfected by nucleofection [27] with siRNAs targeting PKCδ mRNA, immediately prior to plating in mass cultures. Control neurons were alternatively transfected without siRNA (−) or with a negative control siRNA (Neg) designed to not match any known rat mRNA
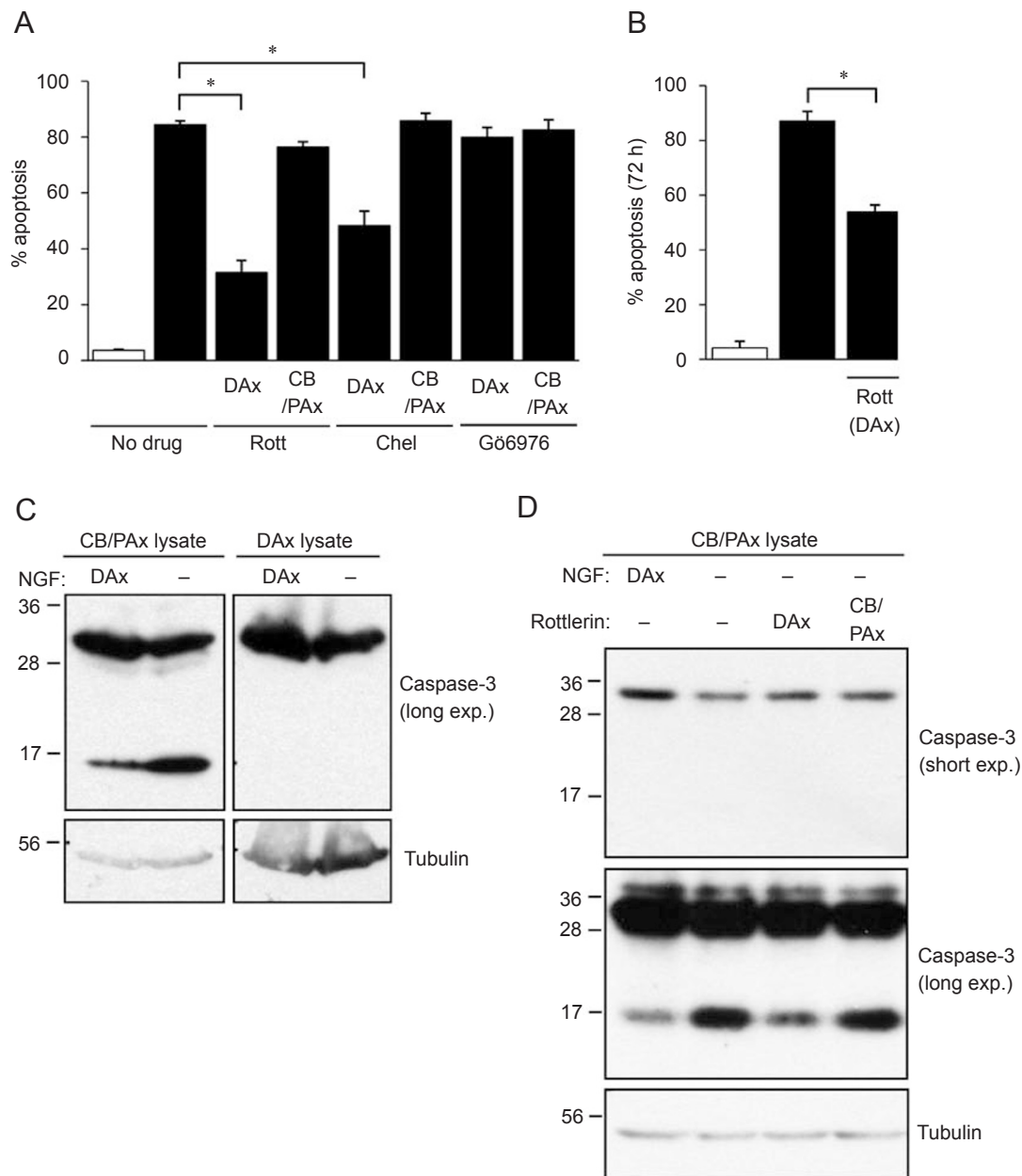
Figure 4 Rottlerin applied to distal axons (DAx) prevents apoptosis and pro-caspase-3 cleavage of sympathetic neurons subjected to complete NGF withdrawal. **(A**, **B)** Cell bodies/proximal axons (CB/PAx) of all cultures were given NGF antibodies while DAx were treated with either 50 ng/ml NGF (white bar) or NGF antibodies (black bars). Concurrently, the PKC inhibitors, rottlerin (Rott, 2.5 μM), chelerythrine (Chel, 10 μM), or Gö6976 (25 nM) were supplied in the compartments indicated. Apoptotic neurons were visually identified by their condensed, fragmented nuclei or absence of nuclei as in Figure 1C. The percentage of apoptotic neurons in each group is plotted (± SEM) from data compiled from three experiments. **(A)** Application of PKCδ inhibitors to DAx, but not to CB/PAx, inhibits apoptosis (*n*≥8 cultures per group, *P<0.001, one-way ANOVA *post-hoc* Scheffe test). **(B)** Rottlerin application to DAx inhibits apoptosis of compartmented cultures deprived of NGF for 72 h (*n*≥5 cultures per group, *P<0.001, one-way ANOVA *post-hoc* Scheffe test). **(C** and **D)** DAx were supplied with NGF (50 ng/ml) or with NGF antibodies (−) where indicated. CB/PAx were treated with NGF antibodies in all cultures. Rottlerin (2.5 μM) was supplied in the specified compartments during treatment. Lysates were analyzed by immunoblot after 21 h of treatment. The blot was probed with an antibody recognizing the p35, full-length pro-caspase-3 and the p17 cleaved form. The cleaved form was only visualized after long exposure times. The blot was reprobed for β-tubulin as a loading control. Results are representative of three independent experiments. **(C)** Pro-caspase-3 cleavage occurs in the CB/PAx but not in DAx during NGF withdrawal. **(D)** Rottlerin application to DAx during NGF withdrawal decreases pro-caspase-3 cleavage.

Figure 5 Rottlerin blocks the axonal apoptotic signal at axon terminals. Sympathetic neurons were cultured in five-compartmented cultures shown in the schematic diagram. After 5 days in culture, NGF was withdrawn from intermediate axons (IAx) and cell bodies/proximal axons (CB/PAx) for 2 days to remove neurons whose distal axons (DAx) had not crossed into the distal compartments. Following this pretreatment, CB/PAx and IAx were given medium containing NGF antibodies and DAx were treated with 50 ng/ml NGF (white bar), NGF antibodies (black bars), and rottlerin (2.5 μM) where indicated for 24 h. Apoptotic nuclei were assessed as in Figure 1C. Data plotted for each group are compiled from three experiments (±SEM, $n \geq 7$ cultures, *$P < 0.05$, **$P < 0.001$, one-way ANOVA *post-hoc* Scheffe test).
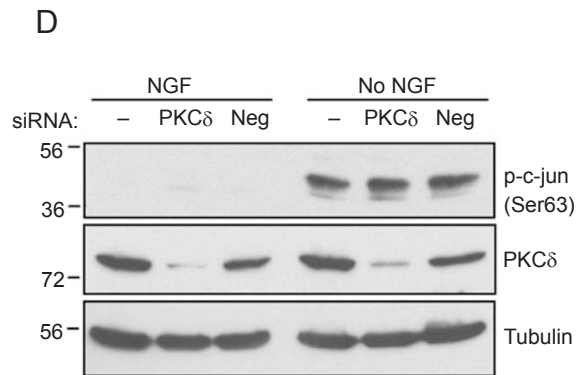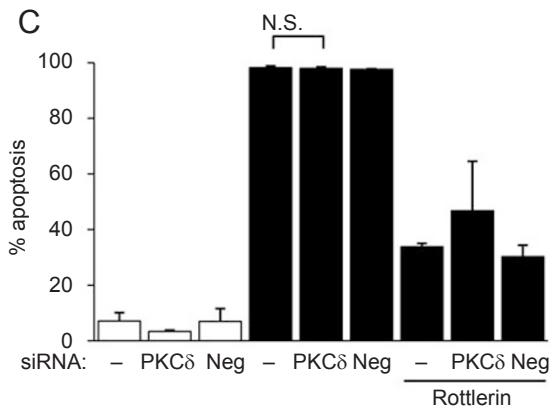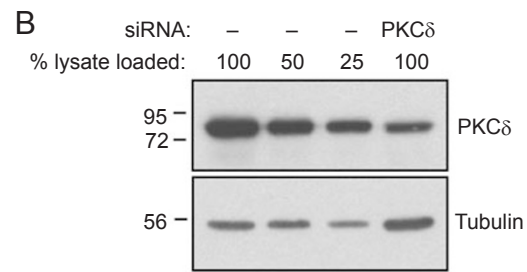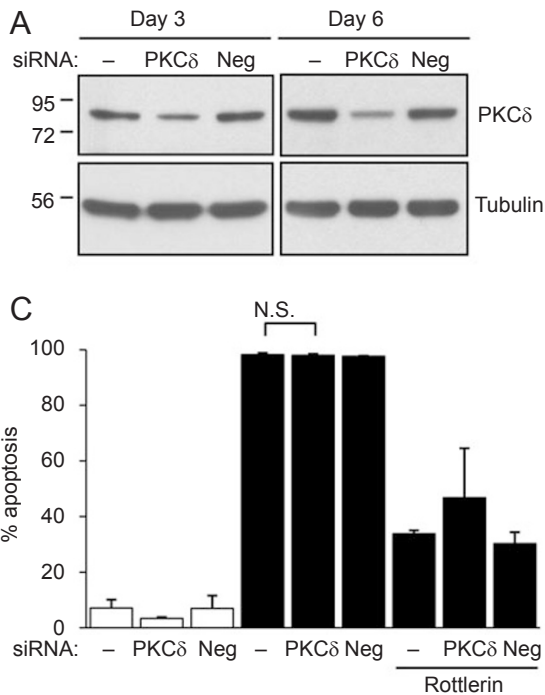
Figure 6 Knockdown of PKCδ by siRNA does not prevent NGF withdrawal-induced apoptosis of sympathetic neurons. Sympathetic neurons in mass cultures were transfected by nucleofection with a pool of four siRNAs (PKCδ) targeting rat PKCδ mRNA prior to plating in mass culture. Transfections were also carried out with no siRNA (−) or a non-specific siRNA (Neg) as controls. **(A)** PKCδ knockdown at the protein level. Transfected neurons were harvested on day 3 or 6 in culture as indicated, and equivalent amounts of cell lysates (8 μg) were analyzed by immunoblot. The blot was probed with an antibody against PKCδ and probed for β-tubulin as a loading control. Results are representative of three independent experiments. **(B)** Quantitation of PKCδ knockdown. Day 4 cultures were harvested and loaded onto an SDS-PAGE gel in the relative amounts indicated (8 μg=100%). The blot was probed for PKCδ and probed for β-tubulin as a loading control. Results are representative of two independent experiments. **(C)** Apoptosis in response to NGF withdrawal. Day 5 transfected cultures were maintained in NGF (white bars) or deprived of NGF (black bars) for 24 h. Rottlerin (2.5 μM) was supplied where indicated. Apoptosis was quantified as in Figure 1C as the percentage of neurons with condensed and fragmented nuclei per group (± SEM, n=4 cultures, N.S.=not significant, one-way ANOVA *post-hoc* Tukey HSD). Data were compiled from two experiments. **(D)** c-jun activation. On day 3 in culture, neurons transfected as indicated, were maintained in NGF (NGF) or deprived of NGF (no NGF) for 6 h, then harvested and analyzed by immunoblot. Blots were probed with an antibody recognizing phosphorylated c-jun (Ser 63). Blots were reprobed for PKCδ and β-tubulin. Results are representative of three independent experiments.

sequences. Immunoblots of 3- and 6-day-old cultures showed a substantial reduction in PKCδ protein levels in neurons transfected with PKCδ siRNA (Figure 6A), estimated by dilution to be greater than 75% (Figure 6B). Transfecting neurons with PKCδ siRNA did not prevent apoptosis (98%) nor did it prevent rottlerin from rescuing NGF-deprived neurons, suggesting that rottlerin's molecular target was still present in neurons with reduced PKCδ (Figure 6C). Immunoblot analysis showed that knockdown of PKCδ did not prevent phosphorylation of c-jun during NGF deprivation (Figure 6D). PKCθ is another PKC isoform inhibited by rottlerin [28]. A cell permeable peptide inhibitor targeting this isoform did not prevent apoptosis induced by NGF withdrawal in mass cultures (Supplementary information, Figure S2). Furthermore, activation of PKCs by phorbol-12-myristate-13-acetate (PMA) did not activate c-jun nor induce apoptosis in NGF-maintained cultures (Supplementary information, Figure S3). These results do not support a role for PKC in triggering the retrograde apoptotic signal.

Rottlerin inhibits activation of GSK3, a kinase that promotes apoptosis of sympathetic neurons during NGF withdrawal

In a further effort to identify rottlerin's protective mechanism, we examined the activation of potential pro-apoptotic molecules. One such molecule, glycogen synthase kinase 3 (GSK3)β, can function in neurons as a pro-apoptotic kinase [29]. GSK3β is also implicated in axon growth [30, 31] and Alzheimer's disease pathology [32]. GSK3β and its closely related isoform GSK3α [33] are regulated by inhibitory phosphorylation (Ser 21 for GSK3α and Ser 9 for GSK3β) [34, 35]. We analyzed the activation state of both GSK3 isoforms by immunoblot in NGF-deprived, rottlerin-treated neurons (Figure 7A). GSK3α and GSK3β were activated (dephosphorylated) in DAx within 6 h of NGF deprivation (compare lanes 5 and 6). Loss of phosphorylation is not caused by decreases in the levels of total GSK3 (Figure 7A). Rottlerin applied to DAx partially blocked the activation of both isoforms in DAx (lane 7), while rottlerin applied to CB/PAx did not prevent activation in DAx (lane 8), suggesting that GSK3 is activated in NGF-deprived DAx by an upstream pathway sensitive to rottlerin.

We next determined whether GSK3 activity promoted apoptosis of NGF-deprived sympathetic neurons. Two inhibitors of GSK3, lithium chloride (LiCl) and kenpaullone [36], block GSK3α and GSK3β activity. Application of LiCl to DAx or CB/PAx during NGF withdrawal (Figure 7B) significantly reduced apoptosis (49% and 46% apoptosis, respectively). Application of LiCl to the entire neuron also reduced apoptosis to 41%, but not sig-

nificantly greater than application to either compartment alone, indicating that the protective effects were not additive. Application of kenpaullone (Figure 7C) to DAx and CB/PAx inhibited apoptosis to 51% and 54%, respectively. However, application of kenpaullone to all compartments (Figure 7C) resulted in a greater protection (33% apoptosis) than either compartment alone suggesting an additive effect. The results with LiCl and kenpaullone suggest that GSK3 activity contributes to apoptosis in response to NGF deprivation. Moreover, the finding that these inhibitors afford protection when applied to DAx or CB/PAx is consistent with the concept that GSK3 plays a role in carrying the retrograde apoptotic signal from DAx to the cell bodies, or that GSK3 activity is required in both DAx and the cell bodies to promote apoptosis. Note that rottlerin applied to DAx may have produced a slight increase in the phosphorylation of GSK3 in CB/PAx (Figure 7A, compare lanes 2 and 3), but this effect was small and is difficult to interpret (see Discussion).

Consistent with possible roles for both GSK3α and GSK3β in retrograde apoptotic signaling, siRNA knockdown of either isoform did not prevent apoptosis of NGF-deprived sympathetic neurons (Supplementary information, Figure S4). However, partial (75% or greater) knockdown of both as determined by immunoblot caused a 27% reduction in apoptosis relative to negative control transfected neurons (Figure 8A and 8B). The incomplete knockdown of GSK3 isoforms may account for the smaller decrease in apoptosis compared with that observed in the GSK3 inhibitor experiments.

Discussion

Previous explanations of how NGF and other neurotrophins promote neuronal survival assume that NGF supports neuronal survival by initiating survival signals at axon terminals that are transmitted retrogradely to the cell bodies [8, 9, 37]. In contrast, our results suggest that NGF at the distal axons also supports survival by suppressing a mechanism in the axons, which in the absence of NGF signaling, produces a retrogradely transported pro-apoptotic signal. This signal is transported to the cell bodies where it initiates the apoptotic program. To obtain this evidence we used two indicators of apoptosis: (1) apoptosis itself as indicated by chromatin condensation and loss of chromatin staining, which are evident at 24 h after NGF withdrawal; and (2) nuclear accumulation of activated c-jun, which occurs within 6 h of NGF withdrawal. c-jun activation was a critical indicator of the initiation of apoptosis in our studies. Several studies from other laboratories have demonstrated that c-jun is required for apoptosis of sympathetic neurons during
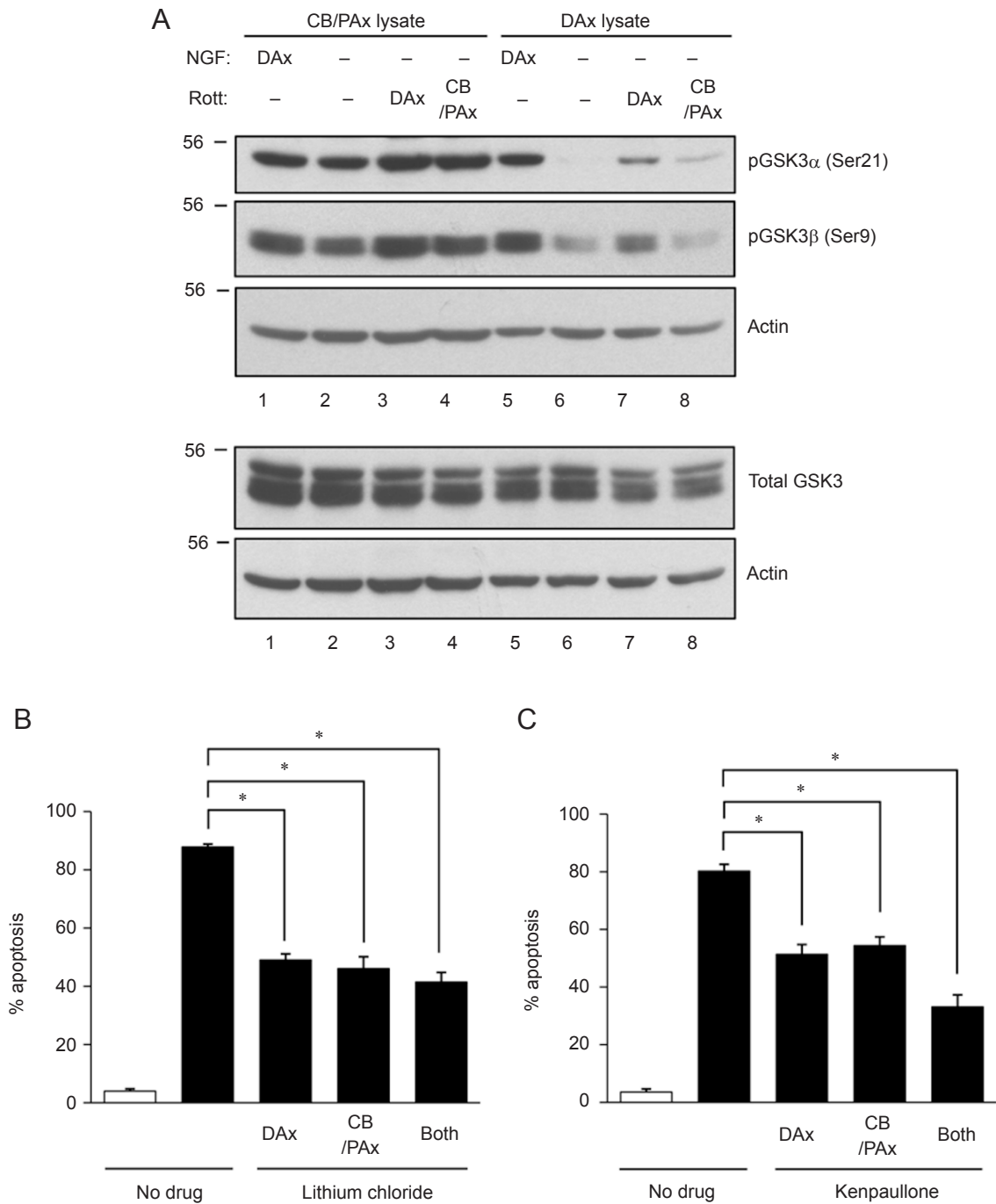
Figure 7 Rottlerin inhibits activation of GSK3, a kinase required for apoptosis in response to NGF withdrawal. **(A)** Rottlerin inhibits activation (dephosphorylation) of GSK3α and GSK3β. Distal axons (DAx) were treated with 50 ng/ml NGF or NGF antibodies (−). Cell bodies/proximal axons (CB/PAx) were treated with NGF antibodies in all cultures. Rottlerin (Rott) was supplied to compartments as indicated. Lysates from each group were split into two equal samples and analyzed on separate immunoblots following 6 h of treatment. One blot was probed with an antibody recognizing phosphorylated GSK3α (Ser 21), reprobed for GSK3β (Ser 9), and then reprobed again for actin as a loading control. The other blot was probed for total GSK3 (both α and β isoforms) then reprobed for actin as a loading control. Results are representative of three independent experiments. **(B, C)** GSK3 inhibitors reduce apoptosis in response to NGF withdrawal. CB/PAx of compartmented cultures received NGF antibodies while DAx were treated with either 50 ng/ml NGF (white bars) or NGF antibodies (black bars). Lithium chloride (15 mM) or kenpaullone (5 μM) was provided to the compartments indicated during treatment. Apoptosis was assessed at 24 h as in Figure 1C and plotted (±SEM, n=9 cultures, *P<0.001, one-way ANOVA *post-hoc* Scheffe test). Results are representative of three independent experiments.
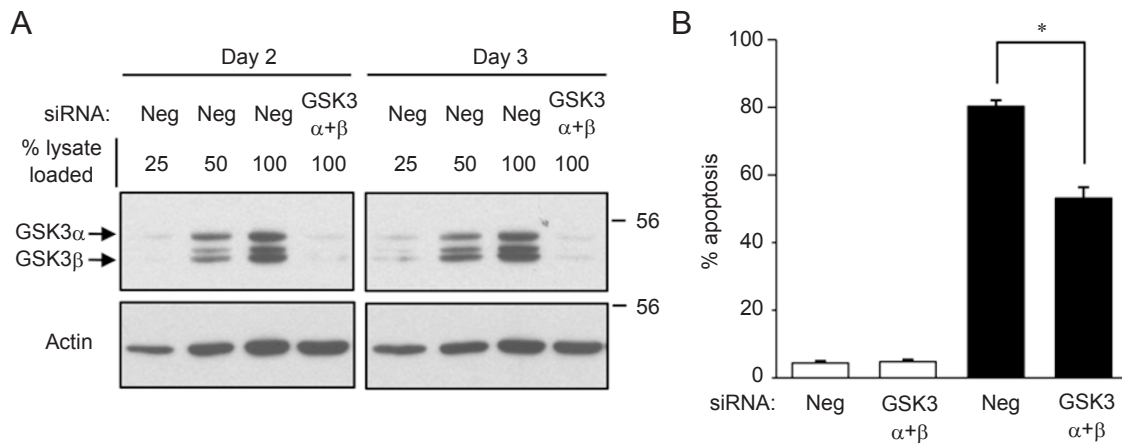
Figure 8 siRNA knockdown of GSK3α and GSK3β decreases apoptosis in response to NGF withdrawal. Sympathetic neurons were transfected by nucleofection with siRNAs targeting rat GSK3α and rat GSK3β mRNA (GSK3α+β) prior to plating in mass culture. Control transfections were also carried out with non-specific siRNA (Neg). **(A)** Quantitation of knockdown. Transfected neurons were harvested on day 2 or day 3 and loaded onto SDS-PAGE gels in the relative amounts indicated (10 μg=100%). The immunoblot was probed with an antibody recognizing total GSK3α and GSK3β. The blot was reprobed for actin as a loading control. Results are representative of three independent experiments. **(B)** Apoptosis in response to NGF withdrawal. On day 2 in culture, transfected neurons were maintained in NGF (white bars) or deprived of NGF (black bars) for 24 h. Apoptosis was quantified as above and plotted (± SEM, n=9 cultures, compiled from three experiments, *P<0.001, one-way ANOVA *post-hoc* Tukey HSD).

NGF withdrawal [17, 18, 38]. Consistent with this, in our cultures, reduction of c-jun protein by siRNA decreased apoptosis of sympathetic neurons in response to NGF withdrawal (data not shown).

The initial clue that NGF withdrawal from the distal axons generates a retrograde apoptotic signal was the observation that c-jun is activated in the cell bodies by withdrawal of NGF from the distal axons, even when neuronal survival is supported by NGF supplied directly to the cell bodies and proximal axons. This was unexpected because neurons supplied with NGF at their cell bodies and proximal axons have an ample supply of NGF-induced survival signals – enough to keep them alive and produce larger NGF-induced alterations in gene expression than observed in neurons supplied with NGF only at their distal axons [39]. In the context of the strong NGF survival signaling generated at the cell bodies and proximal axons, how could the loss of NGF survival signaling from the distal axons produce nuclear accumulation of c-jun as robust and persistent as seen in neurons completely deprived of NGF? And if it did, why wasn't the nuclear accumulation of c-jun sufficient to initiate apoptosis and kill the neurons? It seems more likely that NGF deprivation at the distal axons produces an apoptotic retrograde signal that fully activates c-jun in the cell bodies which initiates the apoptotic program, but that survival signaling generated by NGF applied to cell bodies/proximal axons blocks the apoptotic program

downstream of c-jun. We further tested this hypothesis by blocking axonal transport in the distal axons with colchicine which strongly inhibited the activation of c-jun in the cell bodies of NGF-deprived neurons. Since the activation of c-jun was dependent upon axonal transport in NGF-deprived distal axons, it appears that a signal traveling retrogradely in the axons was responsible for activating c-jun rather than the loss of a signal that had been traveling retrogradely in the axons when they were supplied with NGF. A pro-apoptotic signal traveling retrogradely at a velocity of 10-20 mm/h [40] could traverse the full length of the axons in our cultures in less than an hour which is well within the 3-6 h time frame of c-jun activation during NGF withdrawal [16]. Colchicine applied to the distal axons caused their disintegration after 12 h of treatment (data not shown). Therefore, we were unable to determine if 24 h treatment with colchicine could prevent apoptosis in NGF-deprived neurons since disintegration would abrogate signaling from the original distal axons, and the remaining axon stumps could possibly send injury signals that stimulate apoptosis.

It should be noted that in the novel mechanism presented, neither NGF nor TrkA need to be retrogradely transported to support neuronal survival which is consistent with the results of previous studies from our laboratory [10-12]. NGF can support neuronal survival by functioning locally in the distal axons to suppress the

retrograde apoptotic signal that triggers c-jun activation required for apoptosis. It is also interesting that once generated in the distal axons, the retrograde apoptotic signal that activates c-jun is not turned off by passage into proximal axons and cell bodies supplied with NGF. This suggests that once it is turned on by the loss of NGF signaling, the retrograde apoptotic signal cannot be turned off by the restoration of NGF signaling.

Our hypothesis that NGF deprivation of the distal axons gives rise to a retrograde apoptotic signal is supported by our identification of pharmacological agents, rottlerin and chelerythrine, that block the retrograde induction of c-jun and apoptosis when applied to distal axons of NGF-deprived neurons, but not when applied to cell bodies and proximal axons. If these kinase inhibitors work by activating a survival signaling mechanism, they should, like NGF, also be able to support survival when applied to the cell bodies and proximal axons. The fact that they work only from the distal axons is consistent with the hypothesis that they block the generation of a retrograde apoptotic signal that is generated in NGF-deprived distal axons. Our results showing that rottlerin's protective actions are localized to the distal axon segments containing axon terminals, and not to intermediate axon segments, suggest that the retrograde apoptotic signal may be generated in or near the axon terminals.

PKCδ activity is inhibited by both rottlerin and chelerythrine; however, siRNA knockdown of PKCδ did not prevent apoptosis or prevent rottlerin from blocking apoptosis of NGF-deprived neurons. We cannot rule out a role for PKCδ since the residual PKCδ in transfected neurons could possibly have been sufficient to generate the apoptotic signal, but the lack of effect of knocking down PKCδ suggests that rottlerin and chelerythrine may produce their effects by inhibiting a different molecule.

We found that NGF deprivation of distal axons activates (dephosphorylates) GSK3 in distal axons which is blocked by rottlerin applied to distal axons but not by rottlerin applied to cell bodies and proximal axons. These results are consistent with a scenario in which NGF deprivation activates GSK3 by a rottlerin-sensitive mechanism in the distal axons. We found that lithium chloride and kenpaullone, which directly inhibit GSK3 activity, both prevented apoptosis when applied to either distal axons or cell bodies/proximal axons which is consistent with a possible role for activated GSK3 in carrying a retrograde apoptotic signal along the axon. Rottlerin applied to distal axons may have produced a slight increase in the phosphorylation of GSK3 in cell bodies/proximal axons (Figure 7A, compare lanes 2 and 3) which may be a result of constitutive retrograde transport of phosphorylated, inactivated GSK3 from the distal ax-

ons supplied with NGF switching to retrograde transport of dephosphorylated, activated GSK3 when the axons were deprived of NGF. However, if GSK3 is transported as a consequence of its activation, this may produce little or no change in the level of phosphorylated GSK3 in the cell bodies/proximal axons. Therefore, the small changes in phosphorylation of GSK3 in the cell bodies/proximal axons are difficult to interpret at this time.

Unlike our results with knockdown of PKCδ, partial knockdown of GSK3 protein levels resulted in a decrease in NGF deprivation-induced apoptosis. It is plausible that the inhibition of apoptosis in siRNA-transfected neurons is incomplete due to insufficient knockdown of GSK3. Although immunoblot analysis suggests at least a 75% reduction in GSK3 protein levels, we do not know whether all neurons have a 75% or greater decrease in GSK3 or if there is a large variation in reduction of GSK3 from neuron to neuron. If there is a variation in GSK3 levels within the neuronal population, only those with loss of GSK3 sufficient to prevent the generation/maintenance of the apoptotic signal would be protected. The results with GSK3 clearly represent the initial groundwork for biochemically defining the retrograde apoptotic signaling system. Other possible candidates for carrying the retrograde apoptotic signal include members of the Stress-Activated Protein Kinase (SAPK) pathway, which have been partially characterized in sympathetic neurons [41-44] and lie directly upstream of c-jun, or c-jun itself.

Importantly, we do not see inhibition of the retrograde apoptotic signal generated in distal axons/terminals as the only mechanism of retrograde survival support by neurotrophins or as mutually exclusive with other possible mechanisms that may be operating within the same neuron. NGF-TrkA complexes can be retrogradely transported and activate positive survival signaling in the cell bodies, a process that is the basis of the "signaling endosome hypothesis" [8]. Our previous work has shown that NGF can activate retrograde survival signals in the distal axons that are downstream of TrkA activation, which support neuronal survival without the concurrent retrograde transport of NGF complexed to activated TrkA [10-12].

It should be noted that TrkA is not the only neurotrophin receptor present in sympathetic neurons. p75NTR is a neurotrophin receptor that has been implicated in promoting the apoptosis of sympathetic neurons *in vivo* [45]. It has been proposed in several studies that p75NTR activation generates pro-apoptotic signals that are suppressed by TrkA signaling and that during development neurons receiving insufficient TrkA signaling due to limiting amounts of NGF are susceptible to p75NTR

death signaling [46, 47]. The neurotrophins, BDNF and NT-4, two of the ligands of p75NTR, are expressed in sympathetic neurons, and it is proposed on the basis of mathematical modeling that their secretion may lead to paracrine signaling that promotes apoptosis of neurons in the context of insufficient TrkA signaling [47]. It is parsimonious to think that the axonal apoptotic signal may be a neurotrophin-activated, p75NTR-based signal, however, BDNF and NT-4 only promote apoptosis when they activate p75NTR at the cell bodies/proximal axons [47]. Moreover, in all of the published work of which we are aware, BDNF or NT-4 must be exogenously added to the medium to stimulate p75NTR-mediated apoptosis in cultured sympathetic neurons. Therefore, the axonal apoptotic signal, we describe, represents a distinct pathway.

What are the possible functions that may be served by a retrograde apoptotic signal generated in NGF-deprived axons? One possibility is that this mechanism may produce a faster apoptotic response than can be achieved, without waiting for survival signals produced by NGF to dissipate. Since the half-life of NGF in sympathetic neurons in compartmented cultures is about 6 h [40], and if a significant fraction of internalized NGF is bound to TrkA in signaling endosomes, which continue to signal until they reach the cell bodies, it is hard to see how the level of NGF in the neurons could be reduced enough after 6 h of NGF deprivation to produce the dramatic accumulation of activated c-jun in the nuclei. The time required to turn over NGF would be even greater in neurons with longer axons. Also, NGF retrograde survival signaling has numerous downstream effects, including activation of pro-survival signaling molecules and changes in gene expression, that result in production of proteins with anti-apoptotic effects [48, 49]. Considerable time may be required to inhibit or degrade these proteins after termination of TrkA signaling. Perhaps the axonal apoptotic signal serves to hasten apoptosis by activating an apoptotic pathway that quickly bypasses residual survival signals to kill the neuron quickly.

Another possible function is that a retrograde apoptotic signal could allow neurons to compare death and survival signals coming from different axon branches. If a neuron had part of its axon terminal branches located in an appropriate target that supplies enough NGF-induced retrograde survival signals to keep the neuron alive, but had other axon terminal branches localized in inappropriate targets that supply little or no NGF, it might be beneficial to the organism to override the survival signals and eliminate the neuron entirely. Thus, a retrograde pro-apoptotic signal generated in NGF-deprived axons could be part of a mechanism whereby the relative magnitude of death and survival signals that are being received from the axons determine the survival of the neuron.

There is no reason to assume that retrograde apoptotic signaling systems operate exclusively in sympathetic neurons during NGF-dependent development. The axonal apoptotic signal may continue to be employed in adult neurons to alert the cell body about axon injury, toxic conditions, and so on. Other examples of axonal apoptotic signals have been identified that contribute to HIV neuropathy and axon injury [50, 51]. The existence of retrograde death signals in axons initiated by neurotrophin deprivation has broad implications for discovering treatments for neurodegenerative diseases and neurotrauma. Activation of the c-jun pathway has been linked to the pathology of both Alzheimer's disease and Parkinson's disease [52, 53]. It will be important to determine how widespread retrograde axonal death signaling systems are and what other stimuli can activate them. The existence of retrograde death signaling systems in axons will certainly change the way in which we examine the life and death of neurons in future research.

Materials and Methods

Sympathetic neuron cultures and treatments

Superior cervical ganglia were isolated from 0-2-day-old Sprague-Dawley rats supplied by the University of Alberta Health Sciences Laboratory Animal Services. Compartmented cultures were constructed with a Teflon divider (Tyler Research Corporation, Edmonton, AB) on a 35-mm dish as described in Figure 1A. Three-compartment dividers have 1-mm barriers separating a 1-mm-wide center compartment from 7-mm-wide distal compartments. Five-compartment dividers have an additional 1-mm-wide intermediate compartment between the center and distal compartments. Neurons were dissociated and plated in center compartments of three- or five-compartmented cultures at a density of 0.4 ganglia per culture as previously described [54]. For mass cultures, neurons were plated in 96-well culture plates (Corning Inc., Corning, NY) at a density of 0.12 ganglia per well. Cultures were maintained in L-15 medium (Invitrogen, Carlsbad, CA) modified as described previously [54]. At plating, NGF (2.5S NGF from Alamone Laboratories, Jerusalem, Israel) was provided in distal compartments and in mass cultures at 50 ng/ml and at 10 ng/ml in cell body compartments. In five-compartmented cultures, the intermediate compartments were given 15 ng/ml NGF. The medium provided to center compartments containing the neuronal cell bodies, and to mass cultures, contained 2.5% rat serum and 280 μM vitamin C (Sigma-Aldrich, St Louis, MO). Distal- and intermediate-compartment media lacked rat serum or vitamin C [55]. During the first week in culture, 10 μM cytosine arabinoside (Sigma-Aldrich) was supplied to center compartments and mass cultures to kill non-neuronal cells. Cultures were used in experiments between 6 and 8 days *in vitro* unless otherwise indicated.

During experimental treatments, mass cultures and center compartments containing cell bodies were always provided medium containing rat serum and vitamin C. Compartments were

supplied with medium containing either 50 ng/ml NGF or 24 nM of antibodies to NGF (Cedar Lane, Hornby, ON). When center compartments were treated with NGF, the slot containing the cell bodies, as well as the dish perimeter, were filled with medium to prevent depletion of NGF during incubation. All inhibitors (Calbiochem, San Diego, CA) except LiCl were prepared as working stocks from powder reconstituted in DMSO: Rottlerin at 1.25 mM, Gö6976 at 12.5 μM, chelerythrine at 5 mM, and kenpaullone at 2.5 mM. Inhibitor stocks were added directly to medium at a dilution of 1:500 to achieve final concentrations. All media used in these experiments contained 0.2% DMSO. For LiCl experiments, LiCl (Sigma-Aldrich, St Louis, MO) was added to the media, while maintaining the original osmolarity. NaCl was added to all other media as a control. For experiments longer than 24 h, the medium in all compartments was replaced every 24 h.

Neuronal survival assays

Following experimental treatments, cultures were rinsed with phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde (Fisher Scientific, Ottawa, ON) in PBS for 15 min at room temperature. Mass cultures and the center compartments of cultures were incubated with 10 μM Hoechst 33258 (Molecular Probes, Eugene, OR) in PBS for 10 min. Stained nuclei were examined using a Leica DMIRE2 microscope (Leica, Richmond Wetzlar, Germany) with a UV filter. In cultures deprived of NGF for 24 h, a neuron was scored apoptotic if it had a condensed nucleus. After 24 h, nuclear condensation was followed by loss of nuclear staining in the majority of apoptotic neurons. Therefore, in cultures deprived of NGF for 48 and 72 h, a neuron was assessed as apoptotic if it lacked a nucleus by UV fluorescence. Individual apoptotic neurons in light images were not always clearly visualized, and we estimated a 5% margin of error in quantitation at 48- and 72-h time points.

Immunocytochemistry

Compartmented cultures were fixed with 4% paraformaldehyde at room temperature for 15 min without removing the Teflon dividers. Cells were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) for 10 min, then incubated in a blocking buffer of 5.5% normal goat serum (Invitrogen) in TBS (tris-buffered saline) with 0.1% Tween-20 (TBS-T) (Fisher Scientific) for 1 h. Phospho c-jun (Ser 63) antibody (Cell Signaling Technology, Danvers, MA), at a dilution of 1:1 500, or tubulin antibody (Sigma-Aldrich) at a dilution of 1:500, was applied to cultures in a buffer of 3% BSA in TBS overnight at 4 °C. Cultures were further processed using a rabbit IgG Vectastain Elite ABC Kit (Vector Labs, Burlingame, CA), and immunoreactivity was visualized by the peroxidase reaction of a DAB peroxidase substrate (Sigma Fast 3,3-diaminobenzidine tablet sets, Sigma-Aldrich). Images were taken with a Nikon Diaphot inverted light microscope equipped with a Nikon D70 digital camera (Nikon Canada, Mississauga, ON). The photographic montage of tubulin-stained neurons was assembled using Canon Photostitch software (Canon Canada, Mississauga, ON).

Immunoblotting of compartmented cultures

Following treatment, cultures were rinsed with ice-cold TBS containing sodium fluoride (Sigma-Aldrich, St Louis, MO) and sodium orthovanadate. For CB/PAx compartments, 10 μl of 3× sample buffer was applied to each culture, and cellular material was

dislodged from the substratum by scraping with a hand-held, gel-loading style micropipette tip, then harvested with a micropipette and pooled in a single sample per group. For distal compartments, each culture was harvested sequentially in 30 μl of 3× sample buffer by scraping using a 200 μl pipette tip, to keep extract volumes low. Material from five cultures was pooled for each group. Extracts were boiled for 5 min, resolved on SDS-PAGE gels, and transferred to Immobilon-P PVDF membranes (Millipore, Billerica, MA). Antibodies recognizing caspase 3 (p35 and p17, 1:1 000), phosphorylated c-jun (Ser 63, 1:1 000), phospho-GSK3α (Ser 21, 1:1 000), and phospho-GSK3β (Ser 9, 1:3 000) were purchased from Cell Signaling Technology. Antibodies recognizing actin (1:2 000) and β-tubulin (1:2 000) were purchased from Sigma-Aldrich. Immunoblots were probed overnight with antibodies at the concentration indicated, and immunoreactivity was detected using enhanced chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce, Rockford, IL).

siRNA transfection and treatments

Pools of siRNAs or individual siRNAs purchased from Dharmacon (Lafayette, CO) were used to target the rat mRNA sequences of PKCδ (SMARTpool siRNA, cat# M-080142-00), GSK3α (ON-TARGETplus siRNA duplex, cat# J-080107-09 and J-080107-11), and GSK3β (ON-TARGETplus SMARTpool siRNA, cat# L-080108-00). Non-targeting siRNA (siCONTROL #1, Dharmacon) was used as a negative control. 200 μM stock solutions of pools/individual siRNAs were made in suspension buffer provided by the manufacturer and used at a final concentration of 18 μM. Superior cervical ganglia were isolated and dissociated normally. Dissociated neurons were transfected using a Nucleofector electroporation apparatus (Amaxa, Cologne, Germany) as described by He et al. [27] modified from Hasaka et al. [56]. Briefly, dissociated cells (about 1 million) were pelleted at 800 × g for 5 min and resuspended in 100 μl of Rat Neuron Nucleofection solution (Amaxa). The cells were then added to a transfection cuvette (Amaxa) containing 10 μl of RNA suspension buffer or siRNA. Cells were transfected on setting G-13, transferred immediately to a warm plating medium (L-15 with 50 ng/ml NGF and rat serum), and plated at a density of 2 ganglia per well (24-well plate) and 0.4 ganglia per well (96-well plate). The plating medium was replaced with a normal culture medium at 16 h, and neurons were grown as described above for mass cultures.

For immunoblotting, 1 well was harvested in a lysis buffer containing protease inhibitors (Complete Mini, Roche, Mannheim, Germany), sodium fluoride (Sigma-Aldrich), and sodium orthovanadate (Sigma-Aldrich) on ice for 30 min. Equivalent amounts of protein for each sample were resolved on 10% SDS-PAGE gels. Immunoblots were probed for PKCδ (1:2 000 Santa-Cruz), phosphorylated c-jun (1:1 000, Cell Signaling Technology), total GSK3 (α and β isoform, 1:1 000, Calbiochem), β-tubulin (1:5 000, Sigma-Aldrich), or actin (1:2 000, Sigma-Aldrich) as indicated. Immunoreactivity was detected using enhanced chemiluminescence (Pierce).

Conjugation of Cy3 to NGF and immunofluorescence imaging

Lyophilized NGF (250 μg) was dissolved in 250 μl of sodium carbonate buffer, pH 9.3, and then mixed with half a pack of Cy3 mono-reactive NHS Esters dye (Amersham) pre-dissolved in 500

µl of the same buffer. The reaction was incubated at room temperature for 60 min. The solution was purified with a NAP-5 gel filtration column (Amersham) equilibrated with a sodium phosphate buffer, pH 7.0, to remove the free Cy3. The concentration of the purified Cy3-NGF was determined according to the manufacturer's directions (Amersham). The Cy3-NGF was applied to compartmented cultures as described for non-labeled NGF. A Leica DMIRE2 microscope was used to visualize Cy3 fluorescence.

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