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Regulation of neuronal survival by the extracellular signal-regulated protein kinase 5

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Abstract

The extracellular signal-regulated protein kinase 5 (ERK5) is a mitogen-activated protein kinase (MAPK) that phosphorylates and regulates various transcription factors in response to growth factors and extra-cellular stresses. To address its biological function during the development of the peripheral nervous system (PNS), we have engineered a novel model of sympathetic neurons in which the *erk5* gene can be deleted *in vitro*. Our data provide for the first time genetic evidence that ERK5 is required to mediate the survival response of neurons to nerve growth factor (NGF). Increased cell death associated with the loss of ERK5 is caused by elevated expression of the BH3-only members of the Bcl-2 family, Bad and Bim. Further investigation indicated that ERK5 suppresses the transcription of the *bad* and the *bim* genes via Ca^{++}/cAMP response element binding protein (CREB) and Forkhead box 03a (Foxo3a), respectively. Consistently, we found that the phosphorylation of both p90 ribosomal S6 kinase (RSK) and protein kinase B (PKB) is impaired in neurons lacking ERK5. Together these findings reveal a novel signaling mechanism that promotes neuronal survival during the development of the PNS.

Keywords

ERK5; MAPK; Bad; Bim; neurons

Introduction

During the development of the brain approximately half of the neurons formed by neurogenesis die by apoptosis before adulthood due to limiting amounts of trophic factors (1). The molecular mechanism underlying this neuronal loss has been greatly facilitated by the phenotypic analysis of nerve growth factor (NGF)-dependent sympathetic neurons lacking specific members of the Bcl-2 family (2). For example, while Bax is essential for NGF withdrawal-induced apoptosis (3), there is functional redundancy between the BH3-only proteins Bad and Bid (4). Unlike *bad*−/− neurons and *bid*−/− neurons that do not exhibit any abnormal phenotype, the targeted deletion of *bim* partially protects sympathetic neurons against trophic factor withdrawal (5). Further studies have shown that increased expression of Bim via the transcription factors c-Jun and Forkhead box 03a (Foxo3a) is critical to trigger neuronal apoptosis (6, 7).

Similarly, *de novo* protein synthesis is a prerequisite for trophic factor-induced survival. This is exemplified *in vivo* by the decreased number of sensory and sympathetic neurons in the brain of *Ca^{++}/cAMP response element binding protein* (*creb*)−/− mice (8). CREB is a

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pro-survival transcription factor that acts as both an activator and a repressor of gene expression in the nervous system (9). The transcriptional activity of CREB is increased upon phosphorylation at serine 133 by numerous protein kinases, including p90 ribosomal S6 kinase (RSK), a downstream target of extracellular-regulated protein kinases 1 and 2 (ERK1/2) and ERK5 (10, 11). The specific requirement of ERK5, but not of ERK1/2, to activate CREB in distally stimulated NGF-dependent sensory neurons provided the first evidence that ERK5 was essential for promoting trophic factor-induced neuronal survival (12). The role of ERK5 in mediating the survival of neurons in the central nervous system via the activation of the myocyte enhancer factor 2 (MEF2) transcription factor has since been reported (13, 14).

ERK5 is a non-redundant mitogen-activated protein kinase (MAPK) stimulated in response to growth factors and cellular stresses via the MAPK kinase 5 (MEK5) (15). Since its cloning in 1995, the lack of biological tools, including specific inhibitors, have made it one of the least studied MAPK subfamilies. Consequently, little is known about the downstream targets of ERK5 and therefore the biochemical mechanisms that mediate the effect of ERK5 remain largely unidentified. To advance the current knowledge, we have developed a novel model of primary cultures of sympathetic neurons in which the *erk5* gene can be deleted *in vitro*. Here we provide genetic evidence that ERK5 is required for neuronal survival by suppressing Bad and Bim expression via CREB and Foxo3a, respectively.

Results

Deletion of the *erk5* gene causes apoptosis

The role of ERK5 in NGF-mediated neuronal survival was examined by testing the effect of *erk5* gene deletion in sympathetic neurons. Homozygous *erk5loxP* SCG neurons were infected with an adenovirus encoding Cre recombinase (Cre) or the green fluorescence protein (GFP) (Figure 1a). Immunofluorescence staining confirmed that adenoviruses at 100 multiplicity of infection (MOI) infected neurons with 100% efficiency (Figure 1a). Genomic PCR analysis using specific primers flanking exon 3 revealed that infection with the Cre virus at 100 MOI for 24 h induced efficient recombination of the *erk5* gene (Figure 1b). This correlated with the complete loss of the ERK5 protein after 48 h, as observed by immunoblot analysis of the cell lysates using a specific antibody to ERK5 (Figure 1b). The retarded migration of ERK5 following SDS-PAGE analysis of wild type extracts was absent when the cells were incubated with the alkaline phosphatase CIP, suggesting that the upper band detected by immunoblot corresponded to a phosphorylated form of ERK5 (Figure 1c). Similarly, the electrophoretic mobility shift was abolished in SCG neurons cultured in the absence of NGF for 15 and 30 min (Figure 1c). The phosphorylation of ERK5 was restored 30 min after the re-addition of NGF (Figure 1c). Together these results demonstrate that ERK5 is phosphorylated in SCG neurons incubated with NGF.

SCG neurons are dependent on trophic support for their survival. This is demonstrated by NGF withdrawal-induced phosphorylation of the pro-apoptotic c-Jun N-terminal protein kinase (JNK) MAPK (Figure 1d), as well as an increased number of nuclei displaying segmented and condensed chromatin (supplementary Figure 1a and b). In addition, caspase 3 activity was elevated with a maximum at 24 h after NGF deprivation (supplementary Figure 1c). Similarly, the absence of ERK5 for 48 h promoted morphological changes in cell shape (Figure 1e) and in chromatin structure (Figure 1f) typical of apoptotic cells, and significantly increased caspase 3 activity (Figure 1g). However, in contrast to NGF withdrawal, the loss of ERK5 did not increase JNK phophorylation (Figure 1d). The level of apoptotic death associated with ablation of ERK5 in the presence of NGF was comparable to that observed with the removal of NGF for 18 h (Figure 1f and g). Control experiments demonstrated that infection of wild type SCG neurons with the Cre virus was not toxic to the

cells (Figure 1h). Together these results indicate that ERK5 is a crucial mediator of the NGF pro-survival signal.

ERK1/2 and protein kinase B (PKB, also known as Akt) have previously been implicated in protecting neurons against stress (17). To establish the relative importance of ERK5, ERK1/2 and PKB in mediating NGF-dependent neuronal survival, we compared the effect of ERK5 deletion with the specific inhibition of ERK1/2 and PKB signaling. Incubation of the cells with either UO126 or wortmannin completely abolished the phosphorylation of ERK1/2 and of PKB at Thr308, but not that of ERK5, demonstrating the specificity of the drugs (Figure 2a). Conversely, ablation of ERK5 by infecting *erk5loxP* SCG neurons with Cre virus did not affect the phosphorylation of ERK1/2 or PKB at Thr308 (Figure 2a). Under conditions of adequate trophic support, loss of ERK5 or inhibition of ERK1/2 or PKB signaling, increased caspase 3 activity to a similar extent ($P>0.05$) (Figure 2b). In contrast to our data, several studies have demonstrated that MEK inhibition has minimal effects on NGF-dependent neuronal survival (18-21). Therefore, to confirm our results, we tested the effect of PD0325901, a novel non-competitive inhibitor of MEK1 with greater potency than UO126 (22). Like UO126, we found that PD0325901 specifically inhibited ERK1/2 phosphorylation (Figure 2c) and decreased the survival of SCG neurons incubated with NGF (Figure 2d). Consistent with the caspase 3 assay (Figure 2b) there was no significant difference in cell death exhibited by SCG neurons infected with Cre or incubated with PD0325901 or with wortmannin (Figure 2d). The effect of functional inhibition of ERK5 and ERK1/2, ERK5 and PKB, or ERK5, ERK1/2 and PKB signaling was additive (Figure 2b and d). Together, these results indicate that the ERK5, ERK1/2, and PKB pathways contribute similarly to the survival of NGF-dependent SCG neurons and that neither of them can fully substitute for the loss of the others.

ERK5 is required to inhibit Bad and Bim expression

To elucidate the mechanism by which the loss of ERK5 causes neuronal apoptosis we investigated the regulation of Bad, whose functional inhibition by ERK5 prevents apoptosis in endothelial cells (23). Immunoblot analysis using a Bad-specific antibody revealed that the expression of Bad was increased by around 3 fold after ablation of ERK5 (Figure 3a). Immunofluorescence studies confirmed that Bad was expressed at a much lower level in control neurons than in neurons lacking ERK5. Indeed, the exposure time used to generate the images was insufficient to detect Bad in LacZ infected cells (Figure 3b). The staining of Bad displayed by Cre infected *erk5loxP* neurons partially co-localized with the mitochondrial heat shock protein 70 (mHsp70) (Figure 3b). This is consistent with the pro-apoptotic function of Bad at the mitochondria where it inhibits the activity of anti-apoptotic Bcl-2 family members (24). Similar increases in the expression of the extra long (EL) and long (L) Bim isoforms were detected following the loss of ERK5 (Figure 3c). In contrast, no marked difference was observed in the levels of Bid expression between GFP- and Cre-expressing *erk5loxP* SCG neurons up until 72 h post-infection (Figure 3d and data not shown). Furthermore, the loss of ERK5 did not cause Bid cleavage (data not shown).

To determine the physiological significance of the increase in *bad* and *bim* expression we tested the effect of down-regulating their expression in *erk5−/−* SCG neurons (Figure 3e and f). Neurons were infected with adenoviruses encoding GFP or Cre 1 h prior to incubating the cells with recombinant shRNA lentivirus targeting the murine *bad* or *bim* genes. A recombinant lentivirus encoding unspecific (ctrl) shRNA was used as a control to monitor the effect of lentivirus infection. Immunoblot analysis indicated that Bad and Bim shRNA efficiently and specifically prevented the increase in Bad and Bim expression caused by *erk5* gene deletion (Figure 3e). The down-regulation of either of these BH3-only proteins prevented the neuronal death associated with the loss of ERK5 (Figure 3f). Together these

results demonstrate that Bad and Bim are required to induce the death of neurons lacking ERK5.

ERK5 controls the transcription of Bad and Bim via CREB and Foxo3a

To determine how ERK5 controls Bad and Bim expression, the level of their transcripts in SCG neurons expressing GFP or Cre was measured by quantitative real time (RT) PCR (Figure 4a). The up-regulation of *bad* mRNA was transient and maximal 18 h after infection with Cre virus, a time which corresponded to a 70% reduction of the *erk5* transcript. Expression of Cre for 36 h was necessary to detect increased *bim* mRNA. Control experiments demonstrated that the transcription of *erk5*, *bad* and *bim* genes was not affected in wild type SCG neurons infected with the Cre virus (data not shown), strengthening our conclusion that ERK5 is required to down-regulate the expression of Bad and Bim under adequate trophic factors conditions.

The transcriptional regulation of Bad and Bim by ERK5 was further examined by luciferase assay using reporter plasmids containing a fragment of the Bad (Bad-Luc) or of the Bim (Bim-Luc) promoter (Figure 4b and c). Increased luciferase activity in SCG neurons following the loss of ERK5 confirmed the negative effect of ERK5 on the *bad* and *bim* promoters (Figure 4b and c). Consistent with the quantitative RT PCR time course analysis (Figure 4a), Bad-luc was activated in neurons 18 h after infection, while 36 h was necessary to detect up-regulation of Bim-luc activity.

Increased activity of Bad-luc and Bim-luc was also observed in differentiated PC6.3 over-expressing a dominant negative (DN) form of ERK5 (Figure 4e and f). Immunoblot analysis using a phospho-specific antibody against ERK5 confirmed that ectopic expression of DN-ERK5 blocked the ability of MEK5 to activate ERK5 (Figure 4d). This correlated with increased Bad and Bim expression. The ability of UO126 and wortmannin to activate Bim-luc (Figure 4f), but not Bad-luc (Figure 4e), demonstrated that ERK1/2 and PKB contribute to down-regulating the expression of Bim, but not of Bad, under adequate trophic factor conditions.

A number of response elements have been identified in the promoters of both the *bad* and the *bim* genes, including putative binding sites for the transcription factors CREB and Foxo3a, which have both been reported to be downstream targets of the ERK5 signaling pathway (12, 25). Bad(mt)-luc and Bim(dm)-luc reporter constructs carrying deletions of the putative CREB and Foxo3a binding sites respectively, were not responsive to the loss of ERK5 (Figure 4b and c). Together, these results indicated that ERK5 suppresses Bad and Bim expression via its ability to stimulate CREB and to inhibit Foxo3a activity, respectively.

ERK5 regulates CREB and Foxo3a activity via RSK and PKB

Next we investigated the mechanism by which ERK5 regulated CREB and Foxo3a activity. A previous study showed that activation of ERK5 in sensory neurons promoted the phosphorylation of CREB via RSK (12). Consistent with this result, we found that SCG neurons lacking ERK5 (Figure 5a) or differentiated PC6.3 over-expressing DN-ERK5 (Figure 5b) exhibited impaired phosphorylation of RSK and CREB. Decreased phosphorylation of CREB in SCG neurons was transient with a maximum at 18 h after *erk5* gene deletion. This indicates that CREB can be phosphorylated in the absence of ERK5 independently of RSK. This compensatory signaling mechanism could explain the transient up-regulation of the *bad* transcript in SCG neurons following the loss of ERK5 (Figure 4a).

Chromatin immunoprecipitation (ChIP) analysis using an antibody to CREB demonstrated that CREB interacted with the *bad*-promoter in differentiated PC6.3 cells incubated with NGF (Figure 5c and d). The DNA purified from the cells was amplified by semi-quantitative

(Figure 5c) or by quantitative RT (Figure 5d) PCR. The amount of PCR product was reduced to that of non-specific binding following expression of DN-ERK5, NGF withdrawal, or mutation of the putative CRE sites. Together, these data indicate that ERK5 suppresses Bad expression by stimulating the transcriptional activity of CREB via RSK and by promoting the binding of CREB to the CRE sites in the *bad*-promoter.

The ability of PKB to prevent increased Bim expression by sequestering Foxo3a in the cytoplasm upon phosphoryation is one mechanism by which PKB promotes neuronal survival (6, 26). In a previous study we have demonstrated that, compared to wild type cells, *erk5−/−* and *mek5−/−* fibroblasts treated with sorbitol display reduced PKB activity which is associated with increased Foxo3a activity (25). Here we found that absence of ERK5 in SCG neurons (Figure 6a) or ectopic expression of DN-ERK5 in differentiated PC6.3 (Figure 6b) specifically prevented the phosphorylation of PKB at Ser473, but not at Thr308. Based on evidence that maximal activation of PKB requires dual phosphorylation at Thr308 and Ser473 (27), this result indicates that the level of PKB activity is lower in neurons lacking a functional ERK5 pathway. Consistently, ChIP analysis using an antibody to Foxo3a demonstrated that Foxo3a interacted with the *bim*-promoter in differentiated PC6.3 cells expressing DN-ERK5 (Figure 6c). Together, these studies suggest that ERK5 down-regulates Bim expression in neurons by a mechanism that implicates PKB-dependent phosphorylation of Foxo3.

Discussion

This study provides genetic evidence that ERK5 mediates the survival response of developing sympathetic neurons to NGF by suppressing the transcription of both the *bim* and *bad* genes (Figure 7). Our hypothesis that ERK5 prevents Bim expression by inhibiting Foxo3a is supported by the demonstration that Foxo transcription factors activate the *bim* promoter in sympathetic neurons deprived of NGF (6). Our previous study showed that decreased Foxo3a activity in *erk5−/−* fibroblasts correlated with a reduced PKB activity compared to wild type cells (25). Here, we found that the phophorylation of PKB at Ser473, but not at Thr308, was impaired in neurons 36 h after the loss of ERK5. Together with recent evidence that the phophorylation of PKB at Ser473 is required for PKB to phosphorylate Foxo3a (28), this study indicates that ERK5 down-regulates Bim expression by promoting PKB-dependent inhibition of Foxo3a (Figure 7). It is interesting to note that the inhibition of PKB signaling by wortmannin further increased the level of neuronal death caused by the absence of ERK5. This is consistent with the idea that defective Ser473 phosphorylation affects only a subset of PKB substrates *in vivo* (28).

Other downstream targets of the ERK5-PKB-Foxo3a signaling pathway include FasL (25). Increased FasL expression enhances apoptosis of ERK5-deficient fibroblasts under conditions of osmotic stress by promoting Bid cleavage (25). In contrast, the death promoting fragment tBid was not detected in *erk5−/−*neurons. Together with evidence that FasL does not contribute to trophic factor deprivation-induced apoptosis of sympathetic neurons (4), this observation suggests that increased FasL is unlikely to be implicated in the death of SCG neurons associated with the loss of ERK5.

Like Bim, we found that increased Bad expression was critical to trigger neuronal apoptosis following the loss of ERK5. Consistent with our data, a previous study has shown that over-expression of Bad in sympathetic neurons overcomes the survival effect of NGF (29). However, the requirement of Bad to mediate the apoptotic response of neurons caused by the loss of ERK5 appears inconsistent with the functional redundancy of Bad with other BH3-only proteins in NGF withdrawal-induced neuronal death (4). This discrepancy can be explained by the fact that, in addition to ERK5, NGF deprivation inhibits ERK1/2 and PKB

activity (17), and stimulates the JNK signaling pathway (30). This leads to additional transcriptional and post-translational modifications of members of the Bcl-2 family which sensitize the cells to apoptotic death. For example, NGF-increased Bcl-2 expression in PC12 cells is blocked following inhibition of ERK1/2 signaling (31). Additionally, upon phosphorylation by JNK, Bim dissociates from the microtubule-associated dynein motor complex and translocates to the mitochondria (32, 33). Thus, mitochondrial translocation of Bim in NGF-deprived neurons exhibiting a low level of Bcl-2 may allow the activation of Bax independently of Bad. This suggests that the transcriptional up-regulation of the *bim* gene may not be sufficient to trigger neuronal death. Consistent with this, we found that elevated Bim expression in SCG neurons lacking ERK5 and in which the level of Bad is down-regulated, is not toxic to the cells. In conclusion, our results support the idea that Bad and Bim are non-redundant BH3-only proteins unless they are post-translationally modified to increase their pro-apoptotic function.

The mechanism underlying the transcriptional regulation of *bad* by ERK5 implicates CREB (Figure 7). This result is strengthened by the finding that CREB binds to the putative Cre sites in the *bad* promoter. CREB activity is regulated by two potential mechanisms. The first is via phosphorylation at Ser133, which increases the transcriptional activity of CREB (34). Evidence that the phosphorylation of CREB and of RSK was impaired in the absence of ERK5 indicates that ERK5 represses *bad* expression via RSK-dependent activation of CREB. This model is supported by the finding that RSK is a substrate of ERK5 (10) and that ERK5 contributes to mediating CREB phosphorylation following neurotrophin stimulation of sensory neurons (12). Furthermore, the decrease in CREB phosphorylation caused by the loss of ERK5 followed the same transient kinetics as that of the up-regulation of the *bad* transcript with a maximum after 18 h. The observation that the level of Bad remains elevated up until 48 h after Cre infection suggests that additional mechanisms increase the stability of the protein. The compensatory signaling pathway that partially restores CREB phosphorylation 24 h after the deletion of the *erk5* gene, allowing repression of the *bad* trancription to resume, is unlikely to implicate ERK1/2 considering that the activity of the *bad* promoter is not affected by UO126. The second mechanism is via the regulation of the binding of CREB to DNA via S-nitrosylation of nuclear proteins that associate with CREB target genes, independently of the phosphorylation of CREB at Ser133 (35). Our findings suggest that ERK5-induced CREB-DNA binding may constitute a mechanism that triggers CREB to act as a repressor of gene expression.

Although ERK5 is required for mediating the survival of sensory (12) and sympathetic (our results) neurons *in vitro*, mice lacking ERK5 in the brain do not display any obvious developmental defect (36). However, the sympathetic and sensory nervous systems were not specifically examined in the animal model. Therefore, a more thorough phenotypic analysis of the mice lacking ERK5 will be required to firmly conclude on the role of ERK5 during brain development. In contrast, ERK5 was shown to be essential for neural differentiation in *Xenopus* early embryonic development (37). This discrepancy between *Xenopus* and mouse models may be explained by the activation of redundant signaling mechanisms in more complex organisms. Although *in vitro* ERK1/2 and PKB are not able to fully compensate for the loss of ERK5, activation of ERK1/2 and PKB may be sufficient to sustain the survival of neurons lacking ERK5 in mice.

The requirement of PKB to maintain SCG neuronal survival in response to NGF has been reported before (18, 38-40). However one study disputes this conclusion (41). Furthermore, most studies have found that inhibition of MEK has minimal effects on NGF-dependent neuronal survival (18-21). One possible explanation for these controversial findings may lie in the difference in the species from which the neurons were prepared (i.e. rats compared to mice) and in the conditions of the cell cultures. This includes the number of days SCG

neurons were kept *in vitro* prior to experimentation, which can influence the signaling mechanisms in these postmitotic neurons. In addition, while other studies have used PD98059 (18-21), we employed UO126 to block ERK1/2 signaling. To confirm our results, we tested the effect of PD0325901, a novel non-competitive inhibitor of MEK1 with greater potency (22). Like UO126, we found that PD0325901 decreased the survival of SCG neurons incubated with NGF. These conflicting results emphasize the advantage of genetic deletion analyses over the use of selective inhibitors which exhibit variability in their efficiency to specifically block the transduction of signals.

The additive effect of *erk5* gene deletion with inhibition of ERK1/2 and PKB signaling *in vitro* suggests that ERK5, ERK1/2 and PKB are components of independent pathways which contribute to the survival of sympathetic neurons via overlapping mechanisms. For example, while suppression of *bad* mRNA expression is specifically controlled by ERK5, *bim* can be transcriptionally regulated by ERK5, ERK1/2 and PKB. In addition, ERK1/2 may be required to maintain Bcl-2 levels (31), and PKB may block the pro-apoptotic function of Bad by phosphorylation (29, 42). The relative importance of these different survival mechanisms to prevent neuronal death is likely to vary depending on the type of stress. For example, ERK1/2, but not PKB, is required to protect SCG neurons against toxic stimuli (43-45). The importance of ERK5 in preventing sympathetic neuronal death due to injury or toxicity remains to be tested. In particular, it will be interesting to determine whether the regulation of the BH3-only protein PUMA, which has been implicated in apoptosis induced by DNA damage in sympathetic neurons independently of JNK (43, 46), is controlled by ERK5. Furthermore, phenotypic analysis of the mice lacking ERK5 in the brain will be required to determine whether ERK5 plays a role in supporting the survival of neurons under certain pathological situations including aging and neurodegenerative diseases.

Materials and Methods

Cell cultures

Sympathetic neurons were obtained from the superior cervical ganglion (SCG) of wild type, *erk5+/loxP* and *erk5loxP/loxP* new born mice (post natal day 0-2), as previously described (47). In brief, sympathetic ganglia were trypsinized for 30 min at 37°C. Single-cell suspensions were purified by preplating for 30 min twice on collagen. Non adherent SCG neurons were collected by centrifugation and cultured for 3 days on poly-L-lysine and laminin coated plates in L15 plating medium containing 3% FBS and 50 ng/ml NGF (Alomone Labs), unless indicated otherwise. All mice employed for this study were hosted in a pathogen-free facility at the University Manchester. Use of animals followed Home Office guidelines and received approval by Manchester University's ethical committee.

The PC6-3 subline of the PC12 cell line were cultured on collagen coated plates in differentiating medium (RPMI containing 1% FBS, 2% horse serum, and 100 ng/ml NGF) to obtain a neuronal phenotype, as previously described (6).

Genotyping of the cells

SCG neurons were incubated overnight at 55°C with proteinase K. The cell lysates were treated with phenol/TE/hydroxyquinolone and the genomic DNA was isolated by precipitation with isopropanol. Genotype determination was performed by PCR using forward (5′-GCTTCTCCCTGTGATGTGAG-3′) and reverse (5′-TGAGCTACGGGCTTTCG-3′) primers. 1300 bp and 250 bp fragments were amplified from the *erk5-flox* and disrupted allele, respectively.

Viral infections

The adenoviruses were amplified in HEK-293-T cells and the viral solution was purified on CsCl gradients. The viral infectivity was determined on HEK-293-T cells. After 3 days in culture, SCG neurons were infected with recombinant adenovirus at 100 MOI. Where indicated, recombinant lentivirus at 50 MOI (Mission^{R} shRNA lentiviral transduction particles, Sigma) was added 1 h later. The cells were cultured in plating medium containing 3% FBS and 50 ng/ml NGF for a further 48 h, unless indicated otherwise.

Immunoblot analysis

Extracts (15-20 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12%, 10% or 8% polyacrylamide gel) and electrophoretically transferred to an Immobilon-P membrane (Millipore, Inc.). Where indicated, the cell lysate was incubated with CIP for 2 h at 37°C prior to being run on SDS-PAGE. The membranes were incubated with 3% non-fat dry milk at 4°C for 30 min and then probed overnight with antibodies to Bad (BD transduction laboratories), Bim (Calbiochem), Bid (R&D Systems), CREB (Cell Signaling Technology), phospho-CREB at Ser133 (Cell Signaling Technology), ERK1/2 (SantaCruz), phospho-ERK1/2 at Thr202 and Tyr204 (Cell Signaling Technology), ERK5 (Upstate Biotechnology), phospho-ERK5 at Thr218 and Tyr220 (Biosource), JNK (BD Pharmingen), phospho-JNK at Thr183 and Tyr185 (Cell Signaling Technology), PKB (Cell Signaling Technology), phospho-PKB at Thr308 or Ser473 (Cell Signaling Technology), RSK (Cell Signaling Technology), phospho-RSK at Thr359 and Ser363 (Cell Signaling Technology), tubulin (Sigma). Immunecomplexes were detected by enhanced chemiluminescence with anti-mouse or anti-rabbit immunoglobulin G coupled to horseradish peroxidase as the secondary antibody (Amersham-Pharmacia).

Immunofluorescence

The cells were fixed in methanol prior to being incubated with specific antibodies to Cre (Chemicon), to Bad (1:100, BD transduction laboratories) or to the mitochondrial heat shock protein 70 (mHsp70) (1:200, Cell Signaling Technology). Immune complexes were detected with secondary anti-mouse and anti-rabbit antibodies conjugated to Texas red (1:500, Invitrogen) or fluorescein (1:500, Jackson ImmunoResearch), respectively. Nuclei were stained with DAPI (5 μg/ml). Fluorescence images were viewed with an Olympus Widefield microscope.

Plasmid constructs

Wild type and double mutant (dm) Bim-luciferase plasmids were provided by J. Ham (6). Wild type and mutant ERK5 were described before (48). The Bad-luciferase construct was created by subcloning fragment −3599 to −1031 of the murine *bad* promoter into pGL3-basic vector (Promega) using XhoI and HindIII. A mutant (mt) Bad-luciferase construct carrying deletion of two putative CRE binding sites at −2291 and at −2714 was generated by overlapping PCR.

Reporter gene expression assay

SCG neurons or PC6.3 cells were transiently transfected using the Metafectene™ reagent (Biontex) with the Bad-, Bim, or CRE-luciferase reporter plasmids together with or without an expression vector encoding DN-ERK5. A pRL-Tk plasmid encoding *Renilla* luciferase was employed for monitoring transfection efficiency. Aliquots of cell lysates were assayed for firefly and *Renilla* luciferase activities according to the manufacturer's instructions (Promega).

Apoptosis assays

SCG neurons were incubated with Hoechst 33342 (5 μg/ml) and propidium iodide (5 μg/ml) to distinguish viable, necrotic and apoptotic cells. Between 100 and 200 neurons per conditions were scored for apoptosis using a Leica DMIL microscope (Leica, Wetzlar, Germany). Only neurons that had clearly segmented and condensed chromatin were counted as apoptotic. For caspase assays cells were lysed in 20 mM HEPES pH 7.5, 100 mM NaCl, 10 mM DTT, 1 mM EDTA containing 0.1% CHAPS and 10% sucrose. Extracts (5-10 μg) were incubated with 200 μM DEVD-AMC caspase 3 specific fluorogenic substrate for 1 h. Cleavage of the substrate was measured by spectrofluorometer. LDH activity was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay for Promega. Briefly, a 50 μl aliquot was removed from the cell medium and incubated with LDH substrate for 30 min at room temperature in the dark. Stop solution was added to terminate the reaction. The presence of metabolised substrate was measured at absorbance 490nm. The amount of LDH detected was proportional to the amount of lysed cells.

Quantitative RT PCR

Total RNA was isolated using the Trizol™ reagent and cDNA synthesis was carried out as previously described (49). RT quantitative PCRs were performed using the SYBR Green I Core Kit (Eurogentec). Primers used were: forward primer, 5′-CTGTGTTCTCTGGCACTCCA-3′ and reverse primer, 5′-TCAGCCACACCCAT ATCAAA-3′ for *erk5*; forward primer, 5′-GCCCCTACCTCCCTACAGAC-3′ and reverse primer 5′-AGGACTTGGGGTTTGTGTTG-3′ for *bim*; forward primer, 5′-CTCCACATCCCGGAACTCTA-3′ and reverse primer 5′- TTAAAGGGACACAGC GATCC-3′ for *bad*; and forward primer, 5′-CCAACTTGATGTATGAAGGCTTTG-3′ and reverse primer 5′-AATTGGTCTCAAGTC AGTGTACAGGC-3′ for *β-actin* to generate amplicons of 100 bp, 84 bp, 107 bp, and 91 bp, respectively. PCR products were detected in the ABI-PRISM 7700 sequence detection systems (Applied Biosystems). Results were analyzed using the $2^{-\Delta\Delta G}$ methods (50). The level of expression of mRNA was normalized to *β-actin* mRNA.

Chromatin Immunoprecipitation assay

PC6.3 cells were transfected with Bad- or Bim-luciferase constructs and subjected to chromatin immunoprecipitation (ChIP) assay (Active Motif) using a CREB antibody (Santa Cruz), a Foxo3a antibody (Upstate Biotechnology), or a control antibody (AKT, cell signaling). Purified immunoprecipitated DNA was subjected to either real time PCR (using the SYBR Green I Core KIT, Eurogentec) or semi-quantitative PCR using the following primer sets: promoter region of *bad* 5′-TTCCTGAGTGGGCCTCATTCCAGCTG-3′ and 5′-CTGTCCTTACACAGTGCCTTC-3′; promoter region of *bim* 5′-TGCCACCAAAGATCTCTACC-3′ and 5′-GCATTTCCTCACAGAGTTGG-3′. Input DNA levels served as loading controls for transfection efficiency

Statistical Analyses

All p values were generated using one or two-way ANOVA analysis, except the data presented in Figure 3a, c and d, where a paired t-test was employed. Ranges were given when the data were obtained from two independent experiments (Figures 2d, 3f, S1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CREB	Ca^{++}/cAMP response element binding protein
ERK	extracellular signal-regulated protein kinase
Foxo3a	Forkhead box O3a
GFP	green fluorescence protein
MAPK	mitogen-activated protein kinase
MEK	MAPK/ERK kinase
NGF	nerve growth factor
PKB	protein kinase B
RSK	p90 ribosomal S6 kinase
SCG	superior cervical ganglion

References

1. Oppenheim RW. Cell death during development of the nervous system. Annu Rev Neurosci. 1991; 14:453–501. [PubMed: 2031577]
2. Ham JH, Yoon CG, Jung KW, Jang JH. BH3-only proteins: key regulators of neuronal apoptosis. Cell Death Differ. 2005; 12:1015–1020. [PubMed: 15933736]
3. Deckwerth TL, Elliott JL, Knudson CM, Johnson EM Jr, Snider WD, Korsmeyer SJ. Bax is required for neuronal death after trophic factor deprivation and during development. Neuron. 1996; 17:401–411. [PubMed: 8816704]
4. Putcha GV, Harris CA, Moulder KL, Easton RM, Thompson CB, Johnson EM. Intrinsic and extrinsic pathway signalling during neuronal apoptosis: lessons from the analysis of mutant mice. J Cell Biol. 2002; 157:441–453. [PubMed: 11980919]
5. Putcha GV, Moulder KL, Golden JP, Bouillet P, Adams JA, Strasser A, Johnson EM. Induction of BIM, a pro-apoptotic BH3-only Bcl2 family member, is critical for neuronal apoptosis. Neuron. 2001; 29:615–628. [PubMed: 11301022]
6. Gilley J, Coffer PJ, Ham J. FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. J Cell Biol. 2003; 162:613–622. [PubMed: 12913110]
7. Whitfield J, Neame SJ, Paquet L, Bernard O, Ham J. Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. Neuron. 2001; 29:629–643. [PubMed: 11301023]
8. Lonze BE, Riccio A, Cohen S, Ginty DD. Apoptosis, axonal growth defects and degeneration of peripheral neurons in mice lacking CREB. Neuron. 2002; 34:371–385. [PubMed: 11988169]
9. Lonze BE, Ginty DD. Function and regulation of CREB family transcription factors in the nervous system. Neuron. 2002; 35:605–2334. [PubMed: 12194863]
10. Ranganathan A, Pearson G, Chrestensen C, Sturgill T, Cobb M. The MAP kinase ERK5 binds to and phosphorylates p90 RSK. Arch Biochem Biophys. 2006; 449:8–16. [PubMed: 16626623]
11. Xing J, Ginty DD, Greenberg ME. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth-factor regulated CREB kinase. Science. 1996; 273:959–963. [PubMed: 8688081]

12. Watson FL, Heerssen HM, Bhattacharyya A, Klesse L, Lin MZ, Segal RA. Neurotrophins use the Erk5 pathway to mediate a retrograde survival response. Nat Neurosci. 2001; 4:981–988. [PubMed: 11544482]
13. Liu L, Cavanaugh JE, Wang Y, Sakagami H, Mao Z, Xia Z. ERK5 activation of MEF2-mediated gene expression plays a critical role in BDNF-promoted survival of developing but not mature cortical neurones. Proc Natl Acad Sci USA. 2003; 100:8532–8537. [PubMed: 12826611]
14. Shalizi A, Lehtinen M, Gaudilliere B, Donovan N, Han J, Konishi Y, Bonni A. Characterization of a neurotrophin signaling mechanism that mediates neuron survival in a temporally specific pattern. J Neurosci. 2003; 23:7326–7336. [PubMed: 12917366]
15. Wang X, Tournier C. Regulation of cellular functions by the ERK5 signalling pathway. Cell Signal. 2006; 18:753–760. [PubMed: 16376520]
16. Martin DP, Schmidt RE, DiStefano PS, Lowry OH, Carter JG, Johnson EM. Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. J Cell Biol. 1988; 106:829–844. [PubMed: 2450099]
17. Kaplan DR, Miller FD. Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol. 2000; 10:381–391. [PubMed: 10851172]
18. Mazzoni IE, Said FA, Aloyz R, Miller FD, Kaplan D. Ras regulates sympathetic neuron survival by suppressing the p53-mediated cell death pathway. J Neurosci. 1999; 19:9716–9727. [PubMed: 10559381]
19. Creedon DJ, Johnson EM, Lawrence JC. Mitogen activated protein kinase-independent pathways mediate the effects of nerve growth factor and cAMP on neuronal survival. J Biol Chem. 1996; 271:20713–20718. [PubMed: 8702822]
20. Klesse LJ, Parada LF. p21 Ras and phosphatidylinositol-3 kinase are required for survival of wild-type and NF1 mutant sensory neurons. J Neurosci. 1998; 18:10420–10428. [PubMed: 9852579]
21. Virdee K, Tolkovsky AM. Inhibition of p42 and p44 mitogen-activated protein kinase activity by PD98059 does not suppress nerve growth factor-induced survival of sympathetic neurones. J Neurochem. 1996; 67:1801–1805. [PubMed: 8863483]
22. Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, Mclauchlan H, Klevernic I, Arthur JSC, Alessi DR, Cohen P. The selectivity of protein kinase inhibitors: a further update. Biochem J. 2007; 408:297–315. [PubMed: 17850214]
23. Pi X, Yan C, Berk BC. Big mitogen-activated protein kinase (BMK1)/ERK5 protects endothelial cells from apoptosis. Cir Res. 2004; 94:362–369. [PubMed: 14670836]
24. Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. Bad, a heterodimeric partner for Bcl-Xl and Bcl-2, displaces Bax and promotes cell death. Cell. 1995; 80:285–291. [PubMed: 7834748]
25. Wang X, Finegan KG, Robinson AC, Knowles L, Khosravi-Far R, Hinchliffe KA, et al. Activation of extracellular signal-regulated protein kinase 5 down-regulates FasL upon osmotic stress. Cell Death Differ. 2006; 13:2099–2108. [PubMed: 16710360]
26. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell. 1999; 96:857–868. [PubMed: 10102273]
27. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA. Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J. 1996; 15:6541–6551. [PubMed: 8978681]
28. Jacinto E, Facchinetti V, Liu D, Soto S, Wei S, Jung SY, et al. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell. 2006; 127:125–137. [PubMed: 16962653]
29. Roberts ML, Virdee K, Sampson CPB, Gordon I, Parone P, Tolkovsky A. The combination of Bcl-2 expression and NGF-deprivation facilitates the selective destruction of the Bad protein in living sympathetic neurons. Mol Cell Neurosci. 2000; 16:97–110. [PubMed: 10924254]
30. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science. 1995; 270:1326–1331. [PubMed: 7481820]

31. Liu Y-Z, Boxer LM, Latchman DS. Activation of the Bcl-2 promoter by nerve growth factor is mediated by the p42/p44 MAPK cascade. Nucleic Acids Res. 1999; 27:2086–2090. [PubMed: 10219080]
32. Lei K, Davis RJ. JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. Proc Natl Acad Sci USA. 2003; 100:2432–2437. [PubMed: 12591950]
33. Putcha GV, Le S, Frank S, Besirli CG, Clark K, Chu B, et al. JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. Neuron. 2003; 38:899–914. [PubMed: 12818176]
34. Gonzalez GA, Yamamoto KK, Fischer WH, Karr D, Menzel P, Biggs W 3rd, et al. A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature. 1989; 337:749–752. [PubMed: 2521922]
35. Riccio A, Alvania RS, Lonze RE, Ramanan N, Kim T, Huang Y, et al. A Nitric oxide signalling pathway controls CREB-mediated gene expression in neurones. Mol Cell. 2006; 21:283–294. [PubMed: 16427017]
36. Hayashi M, Lee J-D. Role of the BMK1/ERK5 signaling pathway: lessons from knockout mice. J Mol Med. 2004; 82:800–808. [PubMed: 15517128]
37. Nishimoto S, Kusakabe M, Nishida E. Requirement of the MEK5-ERK5 pathway for neural differentiation in *Xenopus* embryonic development. EMBO rep. 2005; 6:1064–1069. [PubMed: 16179948]
38. Crowder RJ, Freeman RS. Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. J Neurosci. 1998; 18:2933–2943. [PubMed: 9526010]
39. Vaillant AR, Mazzoni I, Tudan C, Boudreau M, Kaplan DR, Miller FD. Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase-Akt pathway to synergistically regulate neuronal survival. J Cell Biol. 1999; 146:955–966. [PubMed: 10477751]
40. Virdee K, Xue L, Hemmings BA, Goemans C, Heumann R, Tolkovsky AM. Nerve growth factor-induced PKB/Akt activity is sustained by phosphoinositide 3-kinase dependent and independent signals in sympathetic neurons. Brain Res. 1999; 837:127–142. [PubMed: 10433995]
41. Philpott KL, McCarthy MJ, Klippel A, Rubin LL. Activated phosphatidylinositol 3-kinase and Akt kinase promote survival of superior cervical neurons. J Cell Biol. 1997; 139:809–815. [PubMed: 9348296]
42. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell. 1997; 91:231–241. [PubMed: 9346240]
43. Anderson CNG, Tolkovsky AM. A role for MAPK/ERK in sympathetic neuron survival: protection against a p53-dependent, JNK-independent induction of apoptosis by cytosine arabinoside. J Neurosci. 1999; 19:664–673. [PubMed: 9880587]
44. Hetman M, Kanning K, Cavanaugh JE, Xia Z. Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. J Biol Chem. 1999; 274:22569–22580. [PubMed: 10428835]
45. Xue L, Murray JH, Tolkovsky AM. The Ras/phosphatidylinositol 3-kinase and Ras/ERK pathways function as independent survival modules each of which inhibits a distinct apoptotic signaling pathway in sympathetic neurons. J Biol Chem. 2000; 275:8817–8824. [PubMed: 10722727]
46. Wyttenbach A, Tolkovsky AM. The BH3-only protein Puma is both necessary and sufficient for neuronal apoptosis induced by DNA damage in sympathetic neurons. J Neurochem. 2006; 96:1213–1226. [PubMed: 16478523]
47. Whitfield J, Neame SJ, Ham J. Methods for culturing primary sympathetic neurons and for determining neuronal viability. Methods Mol Biol. 2004; 282:157–168. [PubMed: 15105563]
48. Seyfried J, Wang X, Kharebava G, Tournier C. A novel MAPK docking site in the N-terminus of MEK5α organizes the components of the ERK5 signaling pathway. Mol Cell Biol. 2005; 25:9820–9828. [PubMed: 16260599]
49. Kayahara M, Wang X, Tournier C. Selective regulation of c-jun gene expression by the mitogen-activated protein kinases via the TPA-responsive element and the myocyte enhancer factor 2 binding sites. Mol Cell Biol. 2005; 25:3784–3792. [PubMed: 15831482]

50. Livak KL, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)). Methods. 2001; 25:402–408. [PubMed: 11846609]

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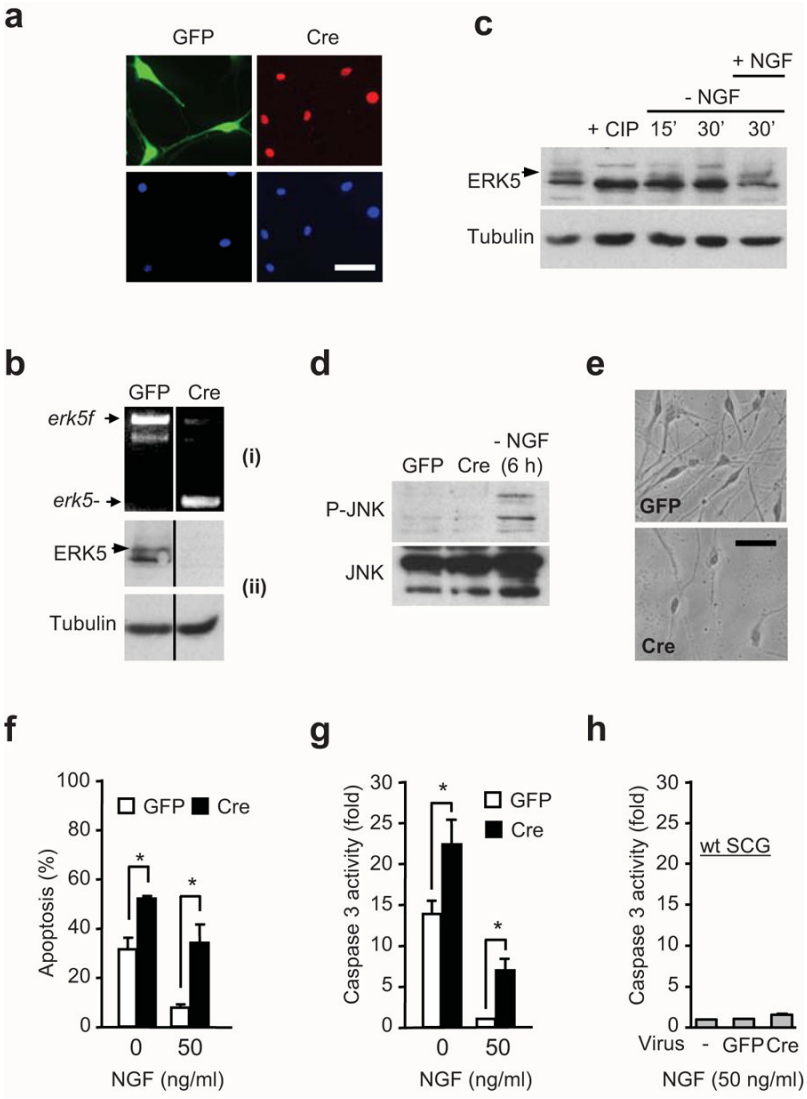

Figure 1.

erk5 gene deletion sensitizes neurons to apoptosis. Homozygous *flox* (*a-g*) or wild type (*h*) SCG neurons were not infected or infected with a control adenovirus (GFP) or with an adenovirus encoding Cre at 100 MOI. The cells were cultured for a further 36 h (*a*) or 48 h (*b*, *d-h*) in presence of NGF (50 ng/ml), unless indicated otherwise. ***a,*** Immunofluorescence analysis of SCG neurons to detect GFP (green) and Cre (anti-Cre antibody, red) expression demonstrates that 100% of the cells were infected by the recombinant adenoviruses. DNA was stained with DAPI (blue). Scale bar, 25 μM. ***b***, (i) Genomic DNA isolated from the cells was amplified by PCR with primers specific for the *erk5* gene. *erk5f* and *erk5-* correspond to the *erk5-flox* and disrupted allele, respectively; (ii) Proteins were extracted and analyzed by immunoblot using specific antibodies to ERK5 and to tubulin. ***c***, Extracts were analyzed for ERK5 expression by immunoblot. The detection of tubulin expression was performed to monitor protein loading. Where indicated, NGF was removed (− NGF) for 15 min and 30 min and re-added (+ NGF) for 30 min, prior to the cells being harvested. CIP treatment of the extract prior to analysis is indicated. Similar results were obtained in two independent experiments. ***d***, Extracts were analyzed for JNK expression and phosphorylation (P) by immunoblot. Where indicated, NGF was removed (− NGF) for 6 h

prior to the cells being harvested. Similar results were obtained in two independent experiments. ***e***, Phase-contrast photomicrographs of representative fields of SCG neurons expressing GFP or Cre are shown. Scale bar, 25 μM. ***f***, SCG neurons were incubated with Hoechst 33342 and propidium iodide to distinguish viable, necrotic and apoptotic cells. Only neurons that had clearly segmented and condensed chromatin were counted as apoptotic. The classification criteria are shown in supplementary Figure 1. ***g***, ***h***, Caspase 3 activity was measured by caspase assay. In some experiments, NGF was removed (0) for 18 h prior to the cells being harvested (*f, g*). The data, expressed as the mean +/− standard error (SE), were generated from three independent experiments performed in duplicate (*f-h*). *, $P \leq 0.001$ indicates a significant difference between GFP and Cre infected neurons. The electrophoretic mobility shift caused by the phosphorylation of ERK5 is indicated by an arrow.

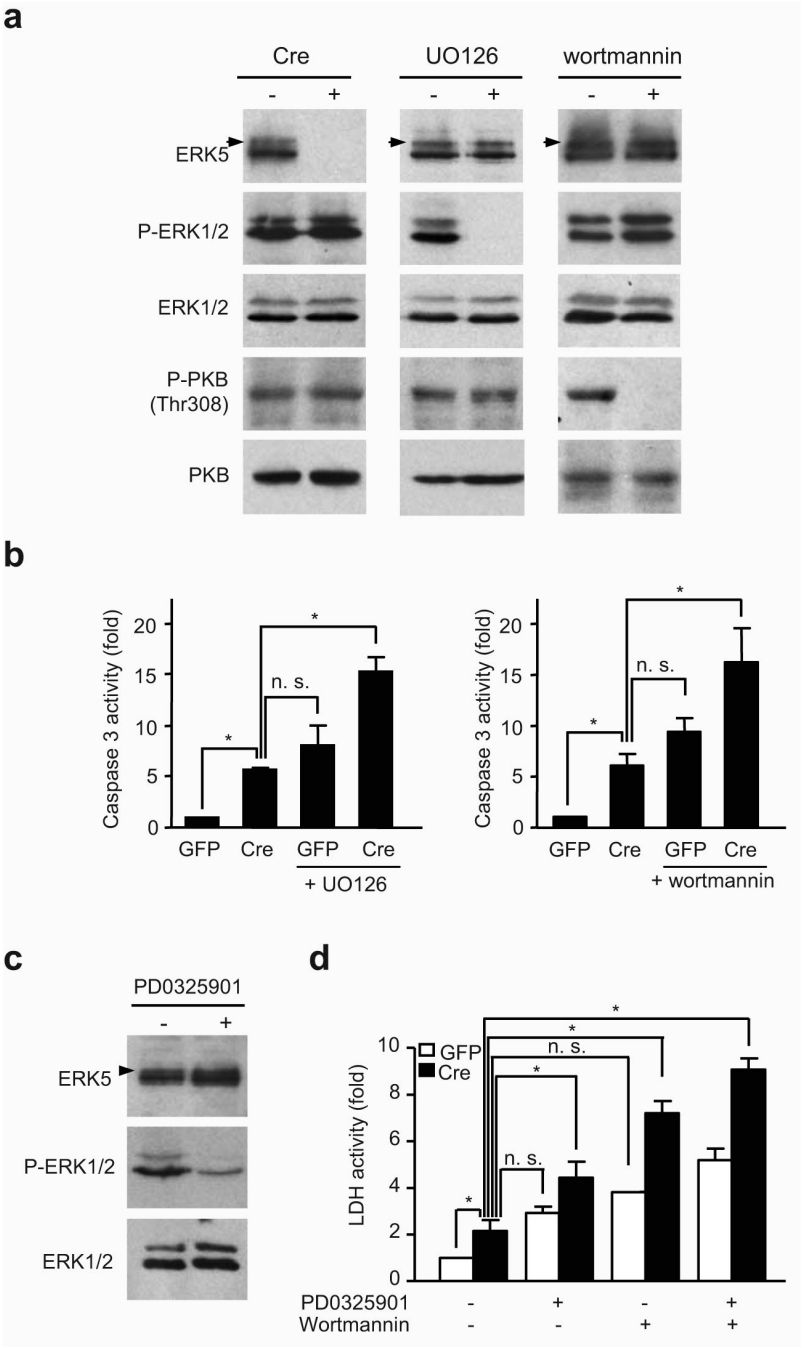

Figure 2.

ERK5, ERK1/2 and PKB are required to support the survival of NGF-dependent SCG neurons. Homozygous *flox* SCG neurons were infected with an adenovirus encoding GFP or Cre. The cells were cultured for a further 48 hours in presence of NGF (50 ng/ml). Where indicated, the cells were treated with UO126 (10 μM), wortmannin (50 nM), or PD0325901 (25 nM) 6 h after the infection. The drugs were replaced every 12 h for the remaining time of the infection. ***a***, ***c***, Extracts were analyzed for ERK5, ERK1/2 and PKB expression, and for phosphorylation (P) of ERK1/2 and of PKB at Thr308 by immunoblot. The electrophoretic mobility shift caused by the phosphorylation of ERK5 is indicated by an

arrow. Similar results were obtained in two independent experiments. ***b***, Caspase 3 activity was measured by caspase assay. The data correspond to the mean ± SE of three independent experiments performed in duplicate. ***d***, Cell survival was measured by LDH assay. The data correspond to the mean ± range of two independent experiments performed in duplicate. *, $P < 0.05$ indicates a significant difference between GFP and Cre infected neurons or between Cre infected neurons treated or not with the inhibitors. n. s., indicates no significant difference ($P > 0.05$).

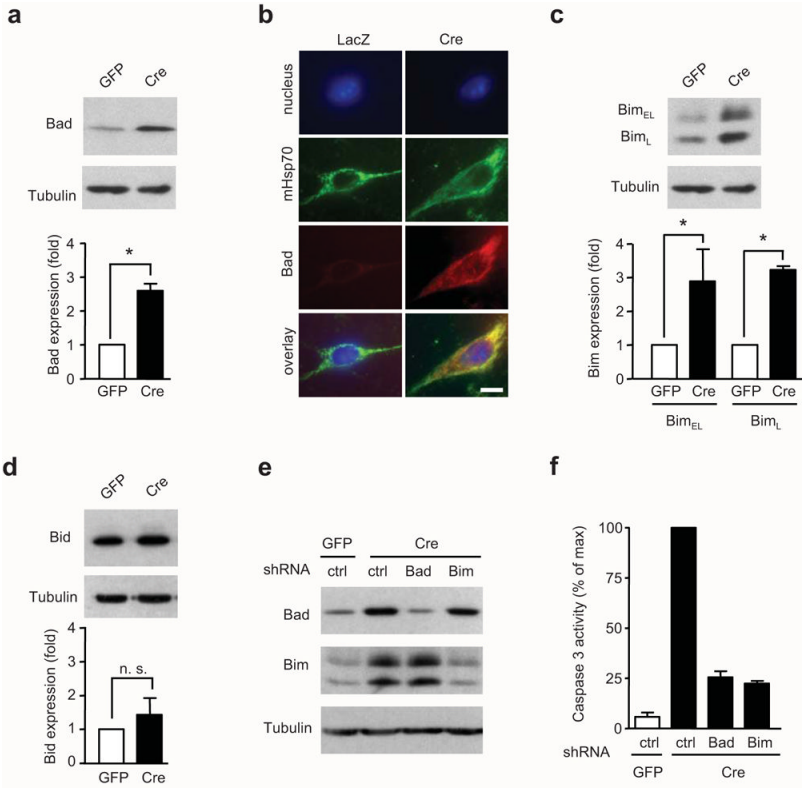

Figure 3.

ERK5 is required to suppress the expression of Bad and Bim. Homozygous *flox* SCG neurons were infected with a control adenovirus (GFP or LacZ) or with an adenovirus encoding Cre. In *e* and *f*, the cells were infected 1 h later with lentiviruses encoding control, bim or bad shRNA. The neurons were cultured for a further 48 hours in presence of NGF (50 ng/ml). ***a***, ***c-e***, Extracts were analyzed for Bad, Bim and Bid expression by immunoblot. The detection of tubulin expression was performed to monitor protein loading. Images of Bad and Bim are from the same samples. Immunoblot signals were quantified with the ImageQuantifier software (BioImage, Jackson MI). The data correspond to the mean ± SE of three independent experiments. *, $P < 0.001$ indicates a significant difference between GFP and Cre infected neurons; n. s., indicates no significant difference ($P > 0.05$). ***b***, Immunofluorescence was performed with specific antibodies to Bad and mitochondrial mHsp70. Immune complexes were detected with secondary antibodies conjugated to Texas red (Bad) or fluorescein (mHsp70). DNA was stained with DAPI (blue). Scale bar, 5 μM. ***f***, Caspase 3 activity was measured by caspase assay. The data correspond to the mean ± range of two independent experiments performed in duplicate.

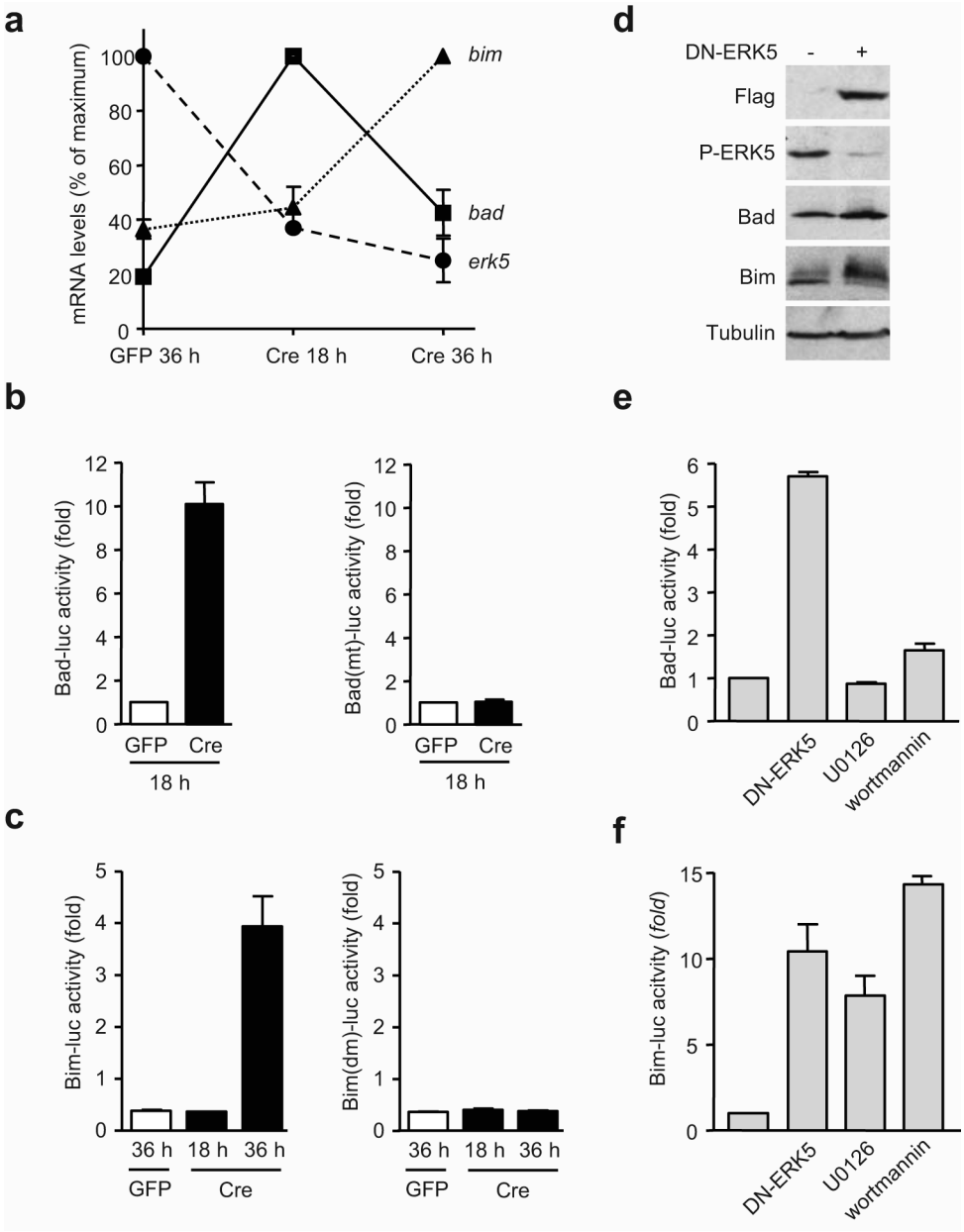

Figure 4.

ERK5 regulates Bad and Bim transcription. ***a***, Homozygous *flox* SCG neurons were infected with an adenovirus encoding GFP or Cre. The cells were cultured in presence of NGF (50 ng/ml) for the indicated times. Total RNA was extracted and the amounts of *erk5*, *bad*, and *bim* transcripts were measured by RT PCR. ***b***, ***c***, Homozygous *flox* SCG neurons were transiently transfected with a Bad or a Bim reporter luciferase plasmid and a pRL-Tk plasmid 20 h or 2 h prior to being infected with an adenovirus encoding GFP or Cre for 18 h and 36 h, respectively. The transcriptional activity was measured by the Dual-Luciferase reporter assay system. ***d-f***, PC6.3 cells were transiently co-transfected with a Bad or a Bim reporter luciferase plasmid and a pRL-Tk plasmid together with (+) or without (−) an expression vector encoding flag-tagged DN-ERK5. The following day the cells were cultured in differentiating medium without or with UO126 (10 μM) or wortmanin (50 nM), for a further 36 hours. The inhibitors were replaced every 12 h. Cell extracts were analyzed

for the expression of DN-ERK5, Bad and Bim, and for the phosphorylation (P) of ERK5 by immunoblot (*d*). The detection of tubulin expression was performed to monitor protein loading. The transcriptional activity was measured by the Dual-Luciferase reporter assay system (*e*, *f*). All the data correspond to the mean ± SE of three independent experiments performed in duplicate.

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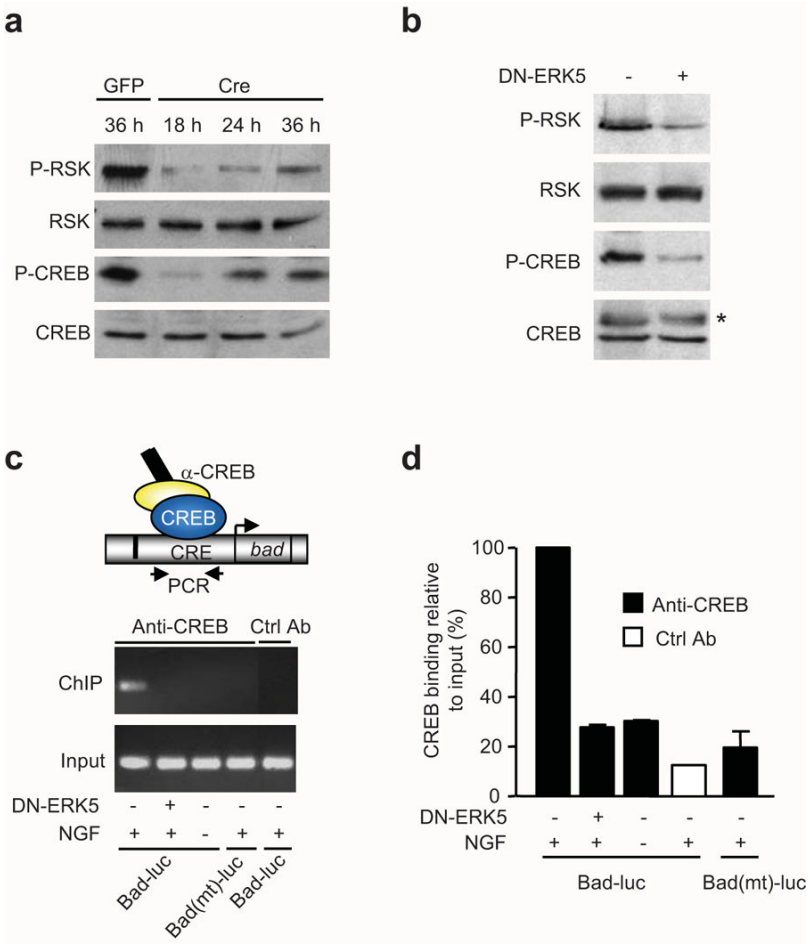

Figure 5.

ERK5 regulates the transcription of *bad* via RSK-dependent CREB phosphorylation. ***a***, Homozygous *flox* SCG neurons were infected with an adenovirus encoding GFP or Cre. The cells were cultured in presence of NGF (50 ng/ml) for the indicated times. ***b-d***, PC6.3 cells were transiently transfected with a wild type or with a CRE-deficient (mt) Bad-luciferase construct together with (+) or without (−) DN-ERK5. The following day the cells were cultured in differentiating medium containing NGF (+) for a further 36 hours. Where indicated, NGF was removed (−) 18 h prior to the cells being harvested. Extracts were analyzed for the expression and the phosphorylation (P) of RSK and of CREB by immunoblot (*a, b*). Chromatin was immunoprecipitated with an antibody to CREB or to an irrelevant protein (Ctrl) to monitor the non-specific binding to the beads. The precipitated DNA was amplified by semi-quantitative PCR (*c*) or by RT PCR (*d*). Input DNA levels were used to monitor transfection efficiency. The data correspond to the mean ± SE of three independent experiments and are normalized to input DNA levels.

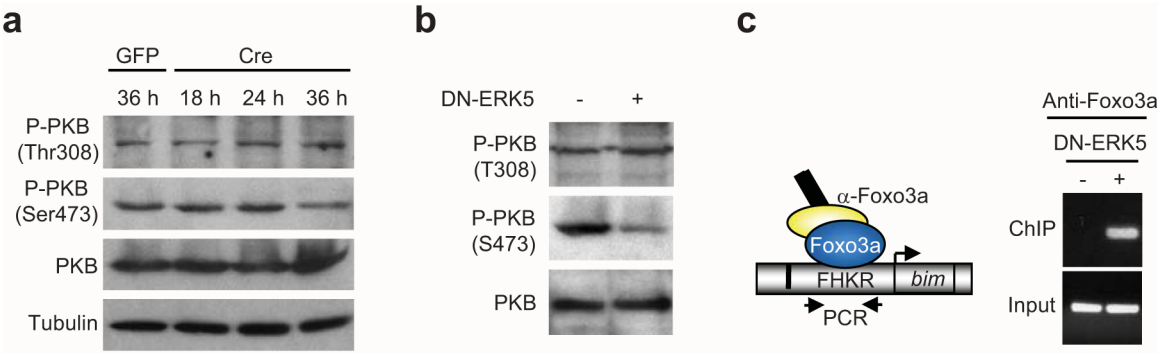

Figure 6.

ERK5 regulates Foxo3a-dependent transcription of *bim* via PKB. ***a***, Homozygous *flox* SCG neurons were infected with an adenovirus encoding GFP or Cre. The cells were cultured in presence of NGF (50 ng/ml) for the indicated times. ***b***, **c**, PC6.3 cells were transiently transfected with (+) or without (−) DN-ERK5. The following day the cells were cultured in differentiating medium containing NGF for a further 36 hours. Extracts were analyzed for the expression and the phosphorylation (P) of PKB at Thr308 or at Ser473 by immunoblot (*a*, *b*). Chromatin was immunoprecipitated with an antibody to Foxo3a. The precipitated DNA was amplified by semi-quantitative PCR (*c*). Input DNA levels were used to monitor transfection efficiency. Similar results were obtained in two independent experiments.

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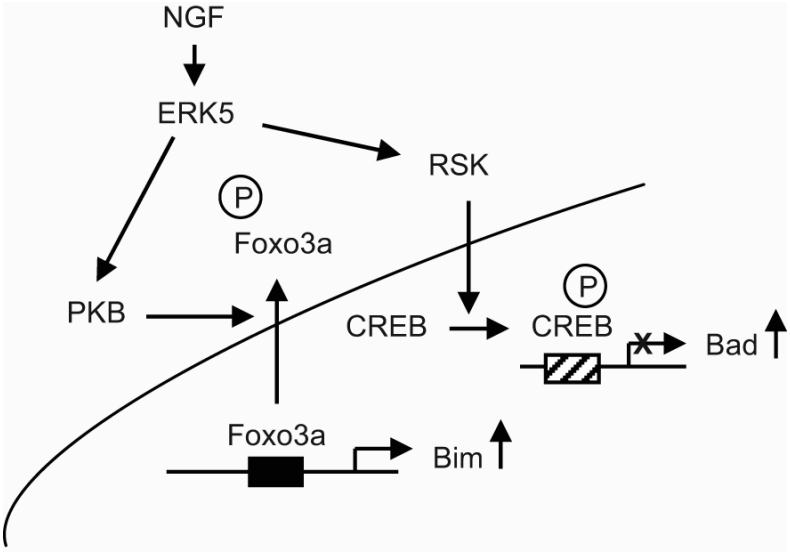

Figure 7.
Regulation of neuronal survival by the ERK5 cascade. The requirement of ERK5 to phosphorylate PKB at Ser473 prevents the nuclear translocation of the pro-apoptotic transcription factor Foxo3a and thereby inhibits Bim expression. ERK5 also mediates the phosphorylation of RSK in response to NGF. In the nucleus, RSK stimulates the transcriptional activity of CREB. CREB is a pro-survival transcription factor that can inhibit the transcription of genes responsible for apoptosis including Bad. The binding of CREB to the CRE sites in the *bad*-promoter is dependent on ERK5.

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