

NRAGE, a p75NTR adaptor protein, is required for developmental apoptosis *in vivo*

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NRAGE (also known as *Maged1*, *Dlxin*) is a member of the *MAGE* gene family that may play a role in the neuronal apoptosis that is regulated by the p75 neurotrophin receptor (p75NTR). To test this hypothesis *in vivo*, we generated *NRAGE* knockout mice and found that *NRAGE* deletion caused a defect in developmental apoptosis of sympathetic neurons of the superior cervical ganglia, similar to that observed in *p75NTR* knockout mice. Primary sympathetic neurons derived from *NRAGE* knockout mice were resistant to apoptosis induced by brain-derived neurotrophic factor (BDNF), a pro-apoptotic p75NTR ligand, and *NRAGE*-deficient sympathetic neurons show attenuated BDNF-dependent JNK activation. Hair follicle catagen is an apoptosis-like process that is dependent on p75NTR signaling; we show that *NRAGE* and p75NTR show regulated co-expression in the hair follicle and that identical defects in hair follicle catagen are present in *NRAGE* and *p75NTR* knockout mice. Interestingly, *NRAGE* knockout mice have severe defects in motoneuron apoptosis that are not observed in *p75NTR* knockout animals, raising the possibility that *NRAGE* may facilitate apoptosis induced by receptors other than p75NTR. Together, these studies demonstrate that *NRAGE* plays an important role in apoptotic-signaling *in vivo*.

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The p75 neurotrophin receptor (p75NTR) is a TNF receptor superfamily member that is expressed at high levels during embryogenesis and functions as a pro-apoptotic receptor during the development of neuronal and non-neuronal tissues.^{1–3} The ligand requirements and downstream signaling cascades activated by p75NTR are beginning to emerge and mounting data indicates that p75NTR induces cell death through mechanisms that are distinct from those activated by other pro-apoptotic TNFR superfamily members. p75NTR-dependent apoptosis does not involve caspase-8 activation but instead requires activation of Jun kinase (JNK), phosphorylation of BH3-domain-only family members, release of mitochondrial contents, and activation of caspase-9.^{4–7}

Independent studies have shown that several members of the *MAGE* protein family bind to the p75NTR cytosolic region^{8–10} and one of them, termed *NRAGE* (also known as *Maged1* and *Dlxin*) is a potent activator of JNK and inducer of apoptosis.^{5,11} *NRAGE* is broadly expressed during development and *in vitro* studies have implicated *NRAGE* in cellular functions ranging from cell cycle regulation and cell adhesion to transcriptional regulation.^{12–15} We have previously shown that *NRAGE* is recruited to p75NTR in a neurotrophin-dependent manner and proposed that *NRAGE* functions as an adaptor protein that links p75NTR to JNK activation.

In this study, we generated *NRAGE* knockout mice to assess the physiological role of *NRAGE* and to specifically test the

hypothesis that *NRAGE* functions as a pro-apoptotic p75NTR adaptor protein *in vivo*. We demonstrate that *NRAGE* plays an essential non-redundant role in developmental apoptosis of sympathetic neurons *in vivo*. In primary sympathetic neurons derived from *NRAGE* knockout mice, apoptosis induced by brain-derived neurotrophic factor (BDNF), a p75NTR ligand, is sharply attenuated. We confirmed these results in rat sympathetic neurons in which *NRAGE* levels were reduced by RNA interference. We also report that *NRAGE* knockout mice exhibit defects in hair follicle catagen that mimic those previously reported for *p75NTR* knockouts. Together, these findings establish that *NRAGE* functions as a pro-apoptotic adaptor protein that mediates p75NTR apoptotic-signaling events *in vivo*. In addition, we show that *NRAGE* knockout mice, but not *p75NTR* knockout mice, have a severe defect in motoneuron apoptosis, suggesting that *NRAGE* may function as an adaptor protein for pro-apoptotic receptors distinct from p75NTR. Taken together, these data demonstrate that *NRAGE* is a versatile pro-apoptotic protein required for developmental apoptosis.

Results

NRAGE knockouts are viable and fertile. To determine if *NRAGE* is required for p75NTR-induced apoptosis *in vivo*, we generated mice in which exons 4–12 of the *NRAGE* gene

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Abbreviations: BDNF, brain-derived neurotrophic factor; HF, hair follicle; JNK, jun kinase; *MAGE*, melanoma antigen-encoding gene; MHD, *MAGE* homology domain; *NRAGE*, neurotrophin-interacting *MAGE* homologue; p75NTR, p75 neurotrophin receptor; SCG, superior cervical ganglion

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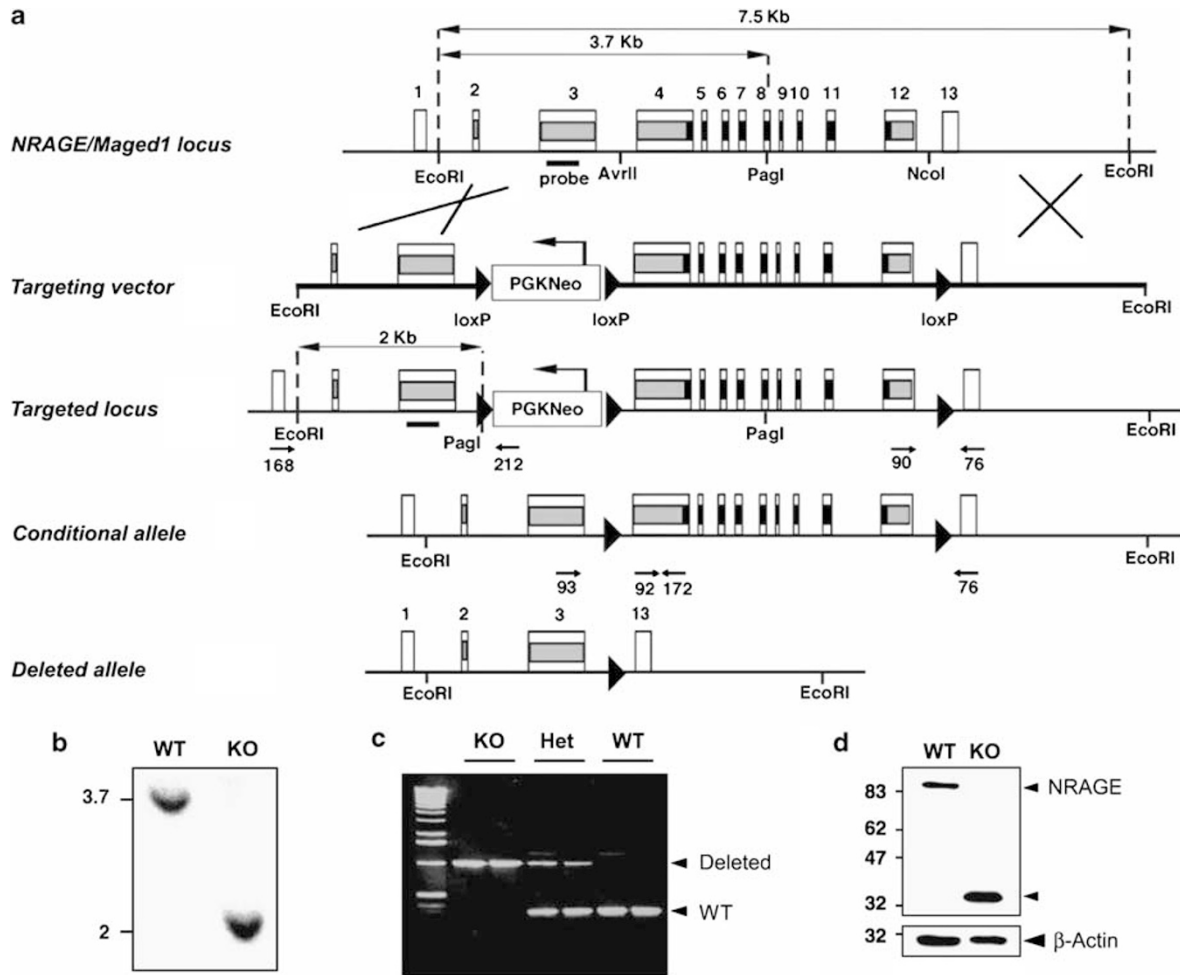


Figure 1 Targeted mutation of *NRAGE/Maged1*: (a) Genomic organization of the *NRAGE/Maged1* locus and schematic representation of the inactivation procedure. The conditional allele (*Maged1^{tm1.Urtm}*) and the deleted allele (*Maged1^{tm1.1Urtm}*), generated after Cre-mediated recombination, are shown. Filled boxes represent the coding region of *NRAGE*, the segment encoding the p75NTR-binding domain is in black. The *loxP* sites are represented by solid triangles. (b) Southern blot analysis of DNA isolated from wild-type ES cells and from ES cells that have undergone gene replacement (*EcoRI/PagI* double digestion). (c) PCR analysis of DNA isolated from tail biopsies of *NRAGE* knockout, heterozygous and wild-type mice. PCR amplification with primers 93 and 76 gives rise to a 940-bp product specific of the deleted allele (*Maged1^{tm1.1Urtm}*) whereas amplification with primers 92 and 172 produces a 270-bp fragment specific of the wild-type allele. (d) Immunoblot analysis of protein lysates from *NRAGE* knockout and wild-type E13.5 brains

were deleted (Figure 1a). The deleted region contains the conserved MAGE homology domain (MHD) as well as the NRAGE segment that binds p75NTR. Southern blotting and PCR confirmed that the *NRAGE* gene was successfully targeted (Figure 1b and c) and immunoblotting of brain lysates demonstrated that full-length NRAGE was absent in the deleted animals. Exons 1–3 of the *NRAGE* gene remained intact in the deleted animals and we observed the presence of a truncated NRAGE fragment on immunoblots that presumably contained the initiator codon in exon 2 and the subsequent coding sequence in exons 2–3 (Figure 1d). This portion of the NRAGE protein has no obvious protein motifs and is poorly conserved and our analyses assumed that the mice lacking exons 4–12 of the *NRAGE* gene represent functional knockouts.

NRAGE knockout mice were born with normal Mendelian frequencies when maintained as an inbred line on the C57Bl6 strain or when outbred to CD1 animals (Figure 2a). Gross

morphological and histopathological features of *NRAGE* knockout mice were normal (data not shown) and weight gain of *NRAGE* knockout mice was not different from wild-type littermates (Figure 2b). *In vitro* studies have raised the possibility that NRAGE may play a role in skeletal development or in cell cycle control yet whole-mount alcian blue/alizarin red staining did not reveal abnormalities in skeletal development (Figure 2c) and cell cycle control of *NRAGE* knockout dermal fibroblasts, analyzed by FACS analysis, was indistinguishable from that observed in dermal fibroblasts from wild-type animals (Supplementary Figure S1). We therefore focused our efforts to determine if NRAGE has a function in developmental apoptosis that is regulated by p75NTR.

Hair follicle catagen is defective in *NRAGE* knockouts. After morphogenesis, hair follicles undergo alternating periods of cell growth (anagen), apoptosis-driven

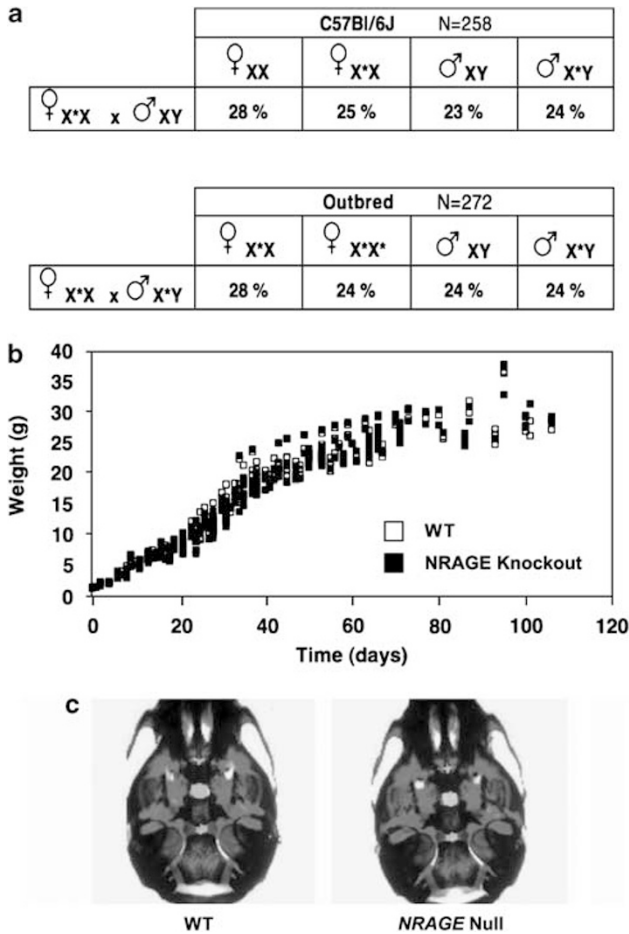


Figure 2 NRAGE deficiency has no impact on viability: (a) The fraction of each genotype generated by crossing heterozygous females (X*X) with either wild-type (XY) or hemizygous (X*Y) males do not differ from Mendelian predictions on either a pure C57Bl/6 background or in outbred CD1 strains. (b) NRAGE knockout and wild-type littermates display similar weight gain. (c) Alcian blue/alizarin red staining of P0 mice did not reveal abnormalities in skeletal development

regression (catagen) and resting (telogen). HF morphogenesis and the first HF catagen and telogen occur with stereotypic developmental timing; in C57BL/6 genetic background, HF morphogenesis is complete by postnatal day 9 (P9), the catagen phase is detected at P17 and telogen starts 3 days later.¹⁶ The apoptosis that occurs in the catagen phase relies on neurotrophin-driven activation of p75NTR.^{17,18} During catagen, most cells expressing p75NTR lie in the outer root sheath (ORS) and loss of p75NTR is associated with a decreased apoptosis in those cells. We used immunostaining to analyze the expression of NRAGE and p75NTR proteins in HF undergoing catagen at P18 in C57BL/6 mice. Figure 3a shows that both proteins co-localize in the ORS, the site of p75NTR-dependent apoptosis. To compare NRAGE and p75NTR knockout mice for defects in catagen, we examined the anagen to catagen transition, during the first postnatal HF cycle and after depilation, using the criteria established by Paus and co-workers.¹⁶ Back skin samples were collected at P17 for the first HF cycle (WT N=6, KO N=6) and 19 days post depilation (WT N=13, KO N=13). NRAGE-deficient mice

showed a profound delay in catagen-dependent dermal thinning, reduction in the hair follicle bulb volume and hair follicle shortening during the first hair follicle cycle (Figure 3b) and after depilation (data not shown). This defect observed in NRAGE nulls did not reflect a delay of HF maturation as the dynamics of HF morphogenesis and growth, analyzed at P1–P2 (WT N=11, KO N=11) and P14 (WT N=4, KO N=4), was undistinguishable between NRAGE wild-type and knockout littermates (Supplementary Figure S2 and data not shown). At P21 (WT N=3, KO N=3), a light delay in catagen completion was still observed in NRAGE knockout animals when compared with wild-type littermates (Supplementary Figure S2).

p75NTR levels vary during the hair follicle cycle, with highest levels observed in the anagen phase, just before the hair follicle initiates regression.¹⁸ To establish if NRAGE mRNA and protein levels are regulated in a similar manner, we performed *in situ* hybridization and immunoblot analyses on dermal sections and lysates. We found abundant NRAGE mRNA expression during all stages of morphogenesis, moderate expression during catagen and no expression during telogen (Figure 3c and data not shown). Consistent with this, expression levels of NRAGE protein are low in telogen, intermediate in catagen and high during morphogenesis, similar to those previously described for p75NTR (Figure 3d). The tight co-expression and co-regulation of p75NTR and NRAGE levels, together with the precise phenocopying of catagen defects in p75NTR and NRAGE knockout mice, support the hypothesis that a p75NTR-NRAGE signaling pathway regulates hair follicle regression *in vivo*.

NRAGE is required for p75NTR-dependent apoptosis of sympathetic neurons.

NGF and BDNF induce neuronal cell death *in vivo* by binding p75NTR and activating apoptotic-signaling cascades. This can be replicated *in vitro*, where BDNF induces p75NTR-dependent apoptosis of sympathetic neurons derived from the superior cervical ganglia (SCG).^{20,21} To determine if NRAGE could have a function in BDNF-induced death of sympathetic neurons, we first examined NRAGE and p75NTR expression levels in SCG during the period of naturally occurring cell death. Figure 4a shows that NRAGE protein is expressed in postnatal day 4 SCGs and that p75NTR protein expression levels do not change in NRAGE knockout mice. To determine if NRAGE lies on the p75NTR apoptotic cascade, we produced primary cultures of SCG sympathetic neurons from NRAGE knockout mice and wild-type littermates and analyzed the induction of apoptosis in these cells following BDNF exposure. Figure 4b shows that in wild-type neurons, BDNF treatment increased sympathetic neuron cell death by approximately fourfold (from 11 to 43%). In NRAGE knockout neurons, the effect of BDNF was significantly reduced, with exposure to the ligand causing only a twofold increase in SCG cell death (from 12 to 22%). Therefore, we conclude that NRAGE has an important, but not exclusive, function in p75NTR-dependent apoptosis activated by BDNF exposure.

To confirm these results, we produced lentivirus-expressing miRNA that targets NRAGE mRNA for destruction and asked whether depletion of NRAGE reduces BDNF-induced killing in

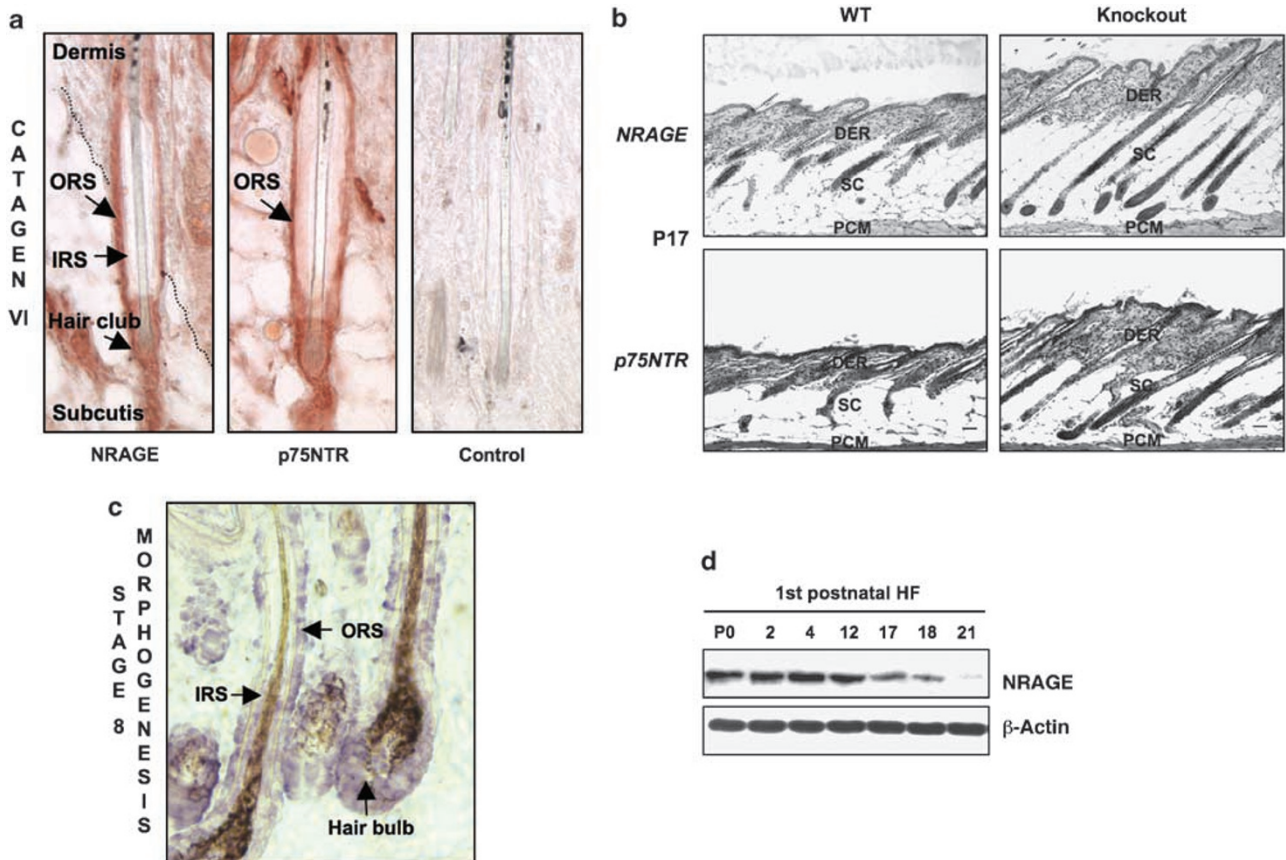


Figure 3 Hair follicle catagen is retarded in *NRAGE* knockout mice: (a) Immunocytochemistry performed on serial back skin sections of a P18 C57BL/6 mouse showing expression of *NRAGE* and *p75NTR* protein in the outer root sheath of a hair follicle on catagen VI. (b) Retardation of hair follicle regression in P17 *NRAGE* knockout mice resembles that observed in P17 *p75NTR* knockout mice. Note the thickening of the dermis in each of the null strains. (c) *In situ* hybridization using an *NRAGE* probe labeled with digoxigenin¹⁹ showing *NRAGE* RNA expression in the inner root sheath, the outer root sheath and hair bulb within a hair follicle of a P4 C57BL/6 mouse. (d) *NRAGE* protein expression at different stages of the first hair follicle cycle. Scale bar in B is 50 μ m. IRS, inner root sheath; ORS, outer root sheath; DER, dermis; SC, subcutis; PCM, panniculus carnosus muscle

primary rat sympathetic neurons. When sympathetic neurons infected with lentivirus that target *NRAGE* mRNA were exposed to BDNF, cell death was dramatically reduced, both at 48 and 72 h after BDNF exposure was initiated (Figure 4c and Supplementary Figure S3). Identical results were obtained using two distinct lentiviruses that target distinct *NRAGE* regions whereas a lentivirus expressing a control miRNA had no effect on BDNF-induced killing.

Activation of JNK is required for sympathetic neurons apoptosis induced by *p75NTR* and we therefore assessed if loss of *p75NTR* or *NRAGE* caused a reduction in BDNF-induced JNK activation in primary sympathetic neurons. Primary rat sympathetic neurons were infected with lentivirus targeting *p75NTR* or *NRAGE* and 6 days later, these cells were examined for BDNF-induced JNK activation. Figure 4d shows that lentivirus targeting *p75NTR* dramatically reduced levels of the receptor (>90%) and that, as expected, BDNF-induced activation of JNK was strongly reduced in these cells. Lentivirus targeting *NRAGE* sharply reduced *NRAGE* protein levels and, importantly, loss of *NRAGE* resulted in an almost complete loss of BDNF-induced JNK activation. We conclude

that *NRAGE* is a key element linking *p75NTR* to JNK activation in sympathetic neurons. As above, identical results were obtained using lentivirus that target different *NRAGE* regions, and the control miRNA had no effect on BDNF-induced JNK activation.

p75NTR has an important role in the developmental apoptosis of sympathetic neurons within the SCG. In mice, the pool of SCG neurons normally declines by more than 50% over the first 3 weeks of life and previous studies have shown that this decrease is significantly attenuated in *p75NTR* knockout animals.^{20,22} To determine if *NRAGE* has a role in the developmental death of sympathetic neurons *in vivo*, we performed neuronal counts on SCGs from wild-type and *NRAGE* knockout mice. At E17, before programmed cell death takes place in these ganglia, the number of sympathetic neurons was essentially identical in wild-type and *NRAGE* knockout littermates. The peak of cell death in the SCG occurs in early postnatal stages and at P3, we found that the number of surviving sympathetic neurons was much higher in *NRAGE* knockout animals. Between P3 and P23, when most programmed cell death has normally ended, the number of sympathetic neurons in the *NRAGE* null animals had dropped

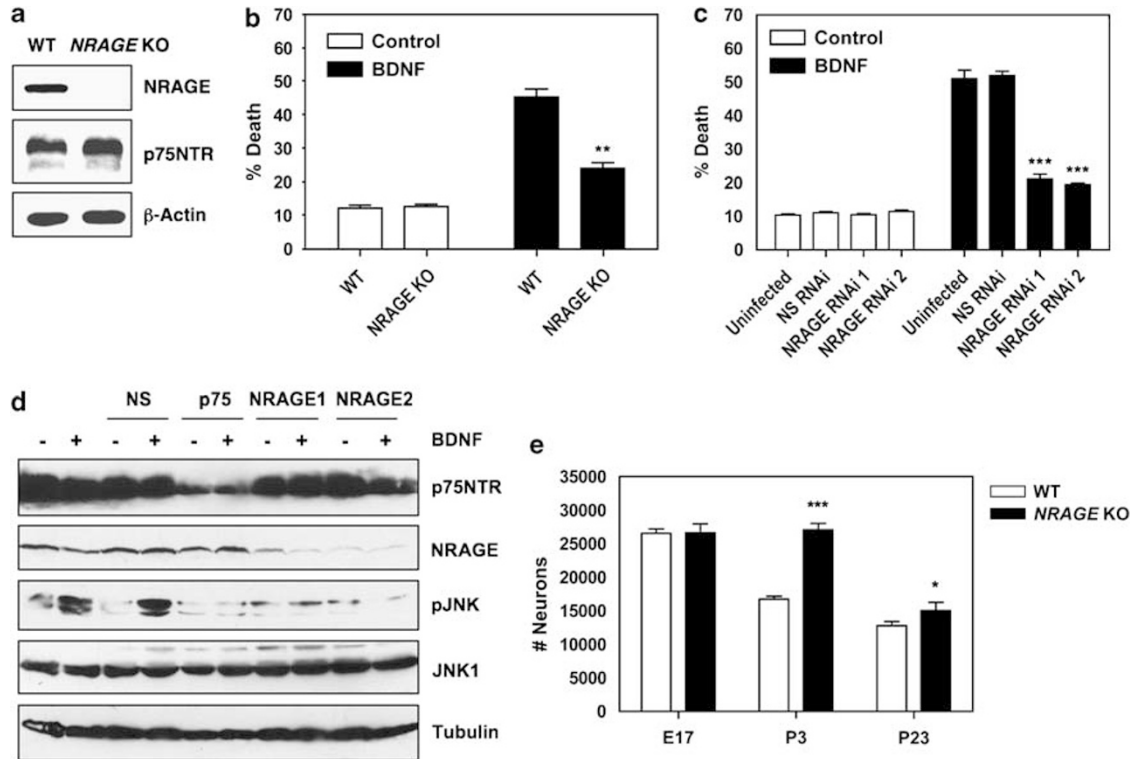


Figure 4 *NRAGE* knockout SCG sympathetic neurons are deficient in p75NTR-induced apoptosis (a) *NRAGE* and p75NTR expression in lysates from P4 SCGs of *NRAGE* knockout and wild-type control pups was determined by immunoblot. (b) Sympathetic neurons derived from *NRAGE* null animals or from wild-type littermates were exposed to BDNF and apoptotic nuclei were quantified 48 h later. Approximately 50–70 neurons were counted for each condition. The experiment was performed in triplicate. ** $P = 0.0022$. (c) Rat sympathetic neurons were infected with lentivirus-expressing miRNA that targets *NRAGE* or were infected with a negative control lentivirus-expressing miRNA that does not target a mammalian protein (NS RNAi), then cultured for 5 days. Neurons were then exposed to BDNF for 72 h and apoptotic nuclei were quantified. *** $P < 0.0001$. (d) Rat sympathetic neurons were infected as in (c) but were also infected with a lentivirus expressing miRNA that targets *p75NTR*. Six days later, neurons were exposed to BDNF for 1 h, lysed and analyzed by immunoblot, as indicated. (e) Number of neurons within the superior cervical ganglia were quantified in *NRAGE* null and wild-type littermates at E17 ($n = 3$), at P3 ($n = 5$) and at P23 (*NRAGE* knockout: $n = 8$, wild-type controls: $n = 10$). *** $P < 0.0001$, * $P = 0.05$. For b, c and e, significance values were calculated using unpaired Student's *t*-tests. Error bars represent S.E.M.

substantially but they were still significantly higher than in wild-type littermates (Figure 4e). We conclude that *NRAGE* is required for normal developmental cell death of SCG sympathetic neurons.

Neuronal growth is normal in *NRAGE* knockout mice. Mice rendered null for p75NTR have defects in neuronal growth that are manifest early in development. To assess whether *NRAGE* contributes to p75NTR-dependent neuronal growth, we compared wild-type, *p75NTR* knockout and *NRAGE* knockout embryos for neuronal growth defects at E11.5, E12.5 and E13.5, using whole-mount immunostaining for β 3-tubulin to mark neuronal projections. As previously reported,^{23–25} *p75NTR* knockout embryos displayed profound defects in several neuronal projections at these stages; as an example, Figure 5 shows that the ophthalmic branch of the trigeminal ganglion is severely truncated in *p75NTR* knockout animals at E12.5. In contrast, neuronal projections in *NRAGE* knockout embryos were indistinguishable from wild-type littermates at all stages examined (Figure 5 and data not shown). Therefore, although *NRAGE* is involved in p75NTR-dependent apoptosis, it is dispensable for effects on neuronal growth.

***NRAGE* knockout mice display defects in developmental apoptosis of motoneurons.** p75NTR is expressed at high levels in developing motoneurons²⁶ and some studies have suggested that NGF facilitates p75NTR-dependent developmental apoptosis in spinal motoneurons.^{27,28} However, the spinal cord motoneuron pool present in wild-type and *p75NTR* knockouts is identical at P7, suggesting that the receptor is not involved in the programmed cell death of these cells *in vivo*.²⁹ Whether p75NTR has a function in motoneuron apoptosis earlier in development has not been examined and we therefore used the whole-mount spread method of Yamamoto and Henderson³⁰ to ask whether p75NTR or *NRAGE* deletion alters developmental death of motoneurons. We found that mice lacking p75NTR do not have a defect in developmental motoneuron death but surprisingly, motoneuron apoptosis was sharply reduced in *NRAGE* null embryos (Figure 6a). Quantification of motoneuron apoptosis in the lumbar region of E13.5 embryos revealed that developmental apoptosis of motoneurons was reduced by 42% in *NRAGE* knockout mice compared with wild-type littermates, a highly significant effect (Figure 6b; $P = 0.008$). These data indicate that *NRAGE* functions in a p75NTR-independent manner to facilitate motoneuron cell death *in vivo*.

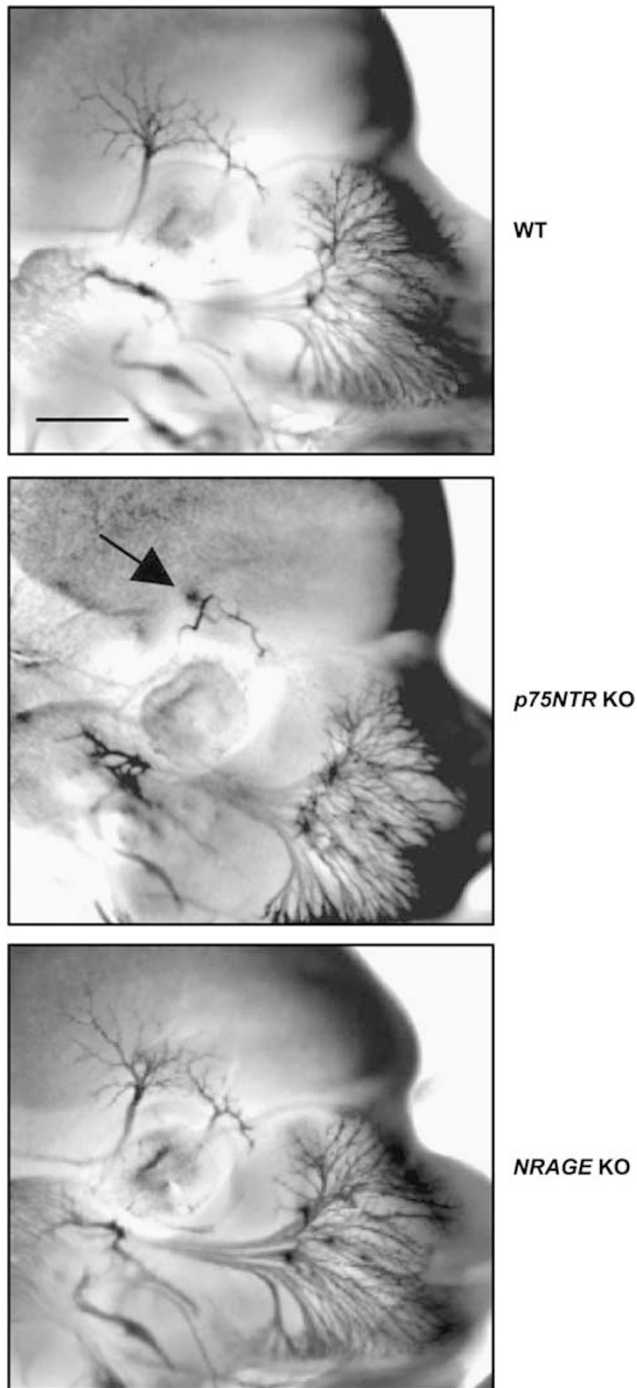


Figure 5 Abnormal outgrowth of the ophthalmic branch of the trigeminal ganglion in *p75NTR* knockout mice but not in *NRAGE* knockout mice. Whole-mount immunostaining of wild-type, *p75NTR* knockout and *NRAGE* knockout embryos with TuJ1, a monoclonal antibody raised against the neuron-specific marker β 3-tubulin. Arrow indicates growth defect in the ophthalmic branch of the trigeminal ganglion in *p75NTR* knockout embryos. Scale bar: 0.35 mm

Discussion

Over 25 *MAGE* genes are expressed in the human genome but their functions remain poorly understood.^{15,31} *MAGE* proteins are characterized by a ~200 amino acid region, of

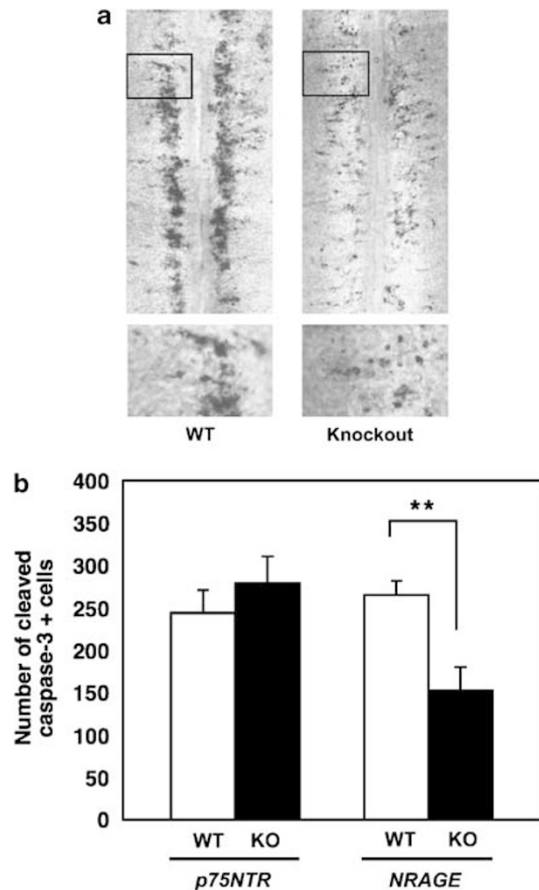


Figure 6 NRAGE, but not *p75NTR*, is required for motoneuron apoptosis *in vivo*. (a) Whole-mount cleaved caspase-3 immunostaining of the lumbar region of E13.5 spinal cords from wild-type and *NRAGE* knockout embryos. Bottom panels represent magnifications of the inset regions. (b) Quantification of lumbar spinal motoneuron apoptosis. *NRAGE* knockout: $n = 4$, wild-type littermates: $n = 5$; *p75NTR* knockout: $n = 8$, wild-type littermates: $n = 12$. $**P = 0.0081$. P -value was calculated using unpaired, two-tailed Student's t -test. Error bars represent S.E.M.

unknown function, termed the *MAGE* homology domain.¹⁵ On the basis of their expression pattern, *MAGE* genes fall into two categories: Type I *MAGE* genes are activated in a wide variety of tumors but are silent in normal tissues, with the exception of testis and, in some cases, the placenta. Type II *MAGE* genes are broadly expressed in embryonic and adult somatic tissues. Most *MAGE* genes are single exon-encoding genes, likely formed by retrotransposition events, and are poorly conserved during mammalian evolution. *NRAGE* (also known as *Maged1* and *Dlxin*) is a Type II *MAGE* gene that shows strong phylogenetic conservation with the unique *MAGE* gene found in *Drosophila* and other lower species,^{32,33} both with regard to its amino acid sequence and its genomic structure. Thus, *NRAGE* is likely to perform a fundamental cellular function, which has been phylogenetically conserved.

NRAGE has been implicated in a large number of cellular processes. *In vitro* studies have indicated that *NRAGE* binds *Dlx* and *Msx* transcription factors and modulates their transcriptional activity^{13,14,34} and that *NRAGE* regulates *p53*-dependent transcription.³⁵ *NRAGE* has also been shown to alter cell cycle progression,³⁶ to disrupt E-cadherin-

dependent adhesion¹² and to facilitate p38 MAPK activation in response to bone morphogenetic proteins.³⁷ Finally, NRAGE binds to the p75NTR intracellular domain and facilitates apoptosis following neurotrophin exposure, suggesting that NRAGE is an adaptor protein that links p75NTR to the apoptotic cascade.^{5,11,38} Our goal in this study was to test the hypothesis that NRAGE functions as an adaptor protein that is required for apoptosis induced by the p75 neurotrophin receptor (p75NTR) *in vivo*.

NRAGE knockout mice are fertile, grossly normal and show no obvious defects at birth or in adulthood. Although NRAGE has been implicated in cell proliferation and in transcriptional events that regulate skeletal development, our analyses did not reveal obvious defects in cell cycle control or in the skeletal system. Given the high degree of homology between NRAGE and the other *MAGED* genes, it is possible that compensatory effects from other alleles dampen an NRAGE loss of function phenotype. The targeted allele produced in this study lacked exons 4–12 (which encodes the MHD as well as the portion of NRAGE that binds p75NTR) but left exons 1–3 intact. The fragment encoded by these exons contain no identifiable protein motifs and does not bind to p75NTR but we cannot rule out the possibility that this fragment has cellular consequences that may ameliorate or exacerbate the consequences of NRAGE gene deletion.

p75NTR has an important function in the developmental cell death of sympathetic neurons. SCGs in mice lacking p75NTR have considerably more sympathetic neurons during and shortly after the period of naturally occurring cell death than their wild-type counterparts.²⁰ We performed neuronal counts of the SCG in NRAGE knockouts and in wild-type littermates and found that NRAGE null mice exhibit a defect in sympathetic neuron apoptosis very similar to that observed in the p75NTR mice. For example, recent results from Ginty and co-workers have indicated that at P3, wild-type mice have about 15 500 SCG neurons whereas p75NTR nulls have approximately 24 000 neurons; we report that P3 wild-type animals have 17 000 SCG neurons whereas the NRAGE null mice have approximately 27 000 neurons. At P21, Deppmann *et al.*²² report that wild-type SCGs have about 12 000 neurons whereas p75NTR nulls have about 15 000 neurons and we report that wild-type animals possess 12 500 *versus* 15 000 neurons in NRAGE nulls. Therefore, SCG survival defects are precisely phenocopied in p75NTR and NRAGE animals, consistent with the hypothesis that a p75NTR-NRAGE-signaling cascade is required for normal SCG neuronal death *in vivo*.

To confirm that NRAGE functions in a p75NTR apoptotic cascade that is activated by neurotrophin, we generated primary sympathetic neuron cultures from NRAGE knockout animals and showed that apoptosis induced by the pro-apoptotic ligand, BDNF, was severely attenuated in these cells. Because NRAGE knockout cells harbor a fragment of the NRAGE gene that may skew these results, we confirmed these findings in primary rat sympathetic neurons, using lentivirus-driving miRNA-targeting NRAGE to knockdown NRAGE gene products. Results obtained using this approach were entirely consistent with data derived from the NRAGE null neurons and we conclude that NRAGE normally has an important function in BDNF-induced apoptosis of sympathetic

neurons. It is noteworthy that NRAGE reduces but does not completely block p75NTR-dependent cell death *in vitro* and therefore independent pro-apoptotic pathways may lie downstream of this receptor. NRIF, has been shown to have an important function in BDNF-induced sympathetic neuronal death *in vitro* and BDNF may therefore activate p75NTR-dependent apoptosis in sympathetic neurons through the actions of at least two adaptor proteins, NRAGE and NRIF. We had previously shown that NRAGE overexpression can activate JNK¹¹ and we therefore tested whether NRAGE knockdown attenuated BDNF-induced JNK activation in sympathetic neurons. We found that knockdown of either NRAGE or p75NTR strongly inhibited JNK activation induced by BDNF, indicating that a p75NTR-NRAGE signaling cascade is required for BDNF-dependent JNK activation in SCG neurons.

We have shown that NRAGE knockout animals show delays in hair follicle catagen that are remarkably similar to those observed in mice lacking p75NTR,^{18,39} suggesting that NRAGE lies on the p75NTR catagen cascade in this organ. Expression analyses showed that NRAGE and p75NTR are co-expressed in the hair follicle compartment where p75NTR-dependent apoptosis occurs and that NRAGE and p75NTR are similarly regulated in this tissue, with increased expression just before catagen. Given that a recent study has shown that proNGF expression peaks during catagen,⁴⁰ it seems likely that the defect in catagen observed in NRAGE knockouts reflects loss of a proNGF-p75NTR-NRAGE signaling cascade.

Approximately one-half of spinal motoneurons initially produced are removed by programmed cell death during development.³⁰ p75NTR is highly expressed in these cells at this stage and several studies have addressed its ability to induce motoneuron cell death. Sedel *et al.*²⁸ found that motoneurons in dissected neural tubes underwent NGF apoptosis that was blocked by antibodies to p75NTR and Wiese and colleagues (1999) showed that administration of exogenous NGF increases p75NTR-dependent death of motoneurons, particularly in injured facial motoneurons.^{27,28} More recently, Pehar *et al.*⁴¹ have shown that NGF can induce apoptosis of normal spinal motoneurons but not those derived from p75NTR knockout mice. Despite these intriguing *in vitro* findings, Murray *et al.*²⁹ found that the pool of spinal cord motoneurons present at P7 is completely normal in p75NTR knockout animals. We examined motoneuron apoptosis during the period of naturally occurring cell death and similarly, found no evidence for a defect in motoneuron apoptosis in p75NTR nulls.

Surprisingly, we found that NRAGE knockout animals show a major defect in the developmental death of spinal motoneurons. The mechanism by which NRAGE participates in programmed cell death in motoneurons are unknown and, although we cannot rule out the possibility that the NRAGE fragment present in knockout animals results in a toxic gain-of-function in motoneurons, a more interesting possibility is that NRAGE functions as an adaptor protein for receptors other than p75NTR to mediate this effect. One interesting candidate for this role is the UNC5A netrin receptor. UNC5A directly binds to NRAGE (through a domain lacking in the NRAGE knockout) and UNC5A induces apoptosis in cultured

cells through an NRAGE-dependent pathway.⁴² Intriguingly, *UNC5A* knockout mice have defects in the developmental apoptosis of motoneurons⁴³ similar to those reported here for the *NRAGE* knockout. The defects in motoneuron apoptosis reported in *UNC5A* knockouts⁴³ are not phenocopied in *Netrin-1* knockouts and the ligand responsible for this effect has not been identified. Identifying the ligand(s) required for *UNC5A*-induced motoneuron apoptosis and assessing the role of NRAGE in this cascade are important future challenges.

To conclude, we have shown that NRAGE is required for p75NTR-dependent apoptosis in sympathetic neurons and required for normal hair follicle catagen, an apoptotic event that is dependent on p75NTR. In addition, we show that NRAGE induces developmental apoptosis of motoneurons, perhaps through functional interactions with other proapoptotic receptors. Taken together, these studies demonstrate that NRAGE is a p75NTR adaptor protein that facilitates programmed cell death *in vivo*.

Materials and Methods

Animal strains. *NRAGE/Maged1* knockout mice are described below. *p75NTR* knockout mice are exon 3-targeted mice originally generated by Dr. Kuo Fee Lee and co-workers.⁴⁴ The morning on which a vaginal plug was observed was considered as E0.5; the day of birth was considered P0. All animal procedures were performed under the guidelines of the Institutional Animal Ethics Committees of the University of Louvain (Belgium), University of Namur (Belgium), IBDM (France) and McGill University (Canada).

Generation of *NRAGE/Maged1*-deficient mice. A conditional allele of *NRAGE* was generated by homologous recombination in ES cells using standard gene targeting techniques. A 7.5-kb *EcoRI* genomic fragment served as a template for the targeting vector. A *loxP* site was introduced into a unique *NcoI* site located in intron 12 by inserting a floxed PGK-*Neomycin* resistance cassette in *Cre*-expressing *E. coli* cells. The same PGK-*Neo* cassette was then inserted into an *AvrII* site located in intron 3 to generate the targeting construct. The vector was linearized using *NotI*, electroporated in E14 ES cells and gene replacement was analysed by PCR (primer 168: 5'-GTCCTTCACCTGGATTCGG-3' and primer 212: 5'-CGAAGTTATACGCTCTCTGAG-3' for the 5' side; and primer 90: 5'-TGACTGGACTGCACAGT-3' and primer 76: 5'-CATGCCACTCTCAGTCAACAGG-3' for the 3' side) and confirmed by Southern blot analysis (*EcoRI/PagI* double digestion) using a probe covering nucleotides 70–642 (AF319975). The *Neomycin* gene was removed by transient expression of *Cre* after electroporation of a plasmid containing a PGK-*Cre* gene and a puromycin-resistance cassette. After a short selection (24 h) with puromycin (2 μ g/ml) and cloning by dilution, ES clones containing the conditional allele (*Maged1^{tm1Urim}*) were identified by PCR (primer 93: 5'-GACCAGCGCAGGTATCTC-3' and primer 172: 5'-TCTCAGATTAGGTGA GGGTCG-3'). Chimeric males were obtained from three independent *Maged1^{tm1Urim}* ES clones after injection into CD1 blastocysts (Eurogentec, Belgium). Heterozygous *Maged1^{tm1Urim}* females were bred with *Cre* deleter males to generate females heterozygous for the knockout mutation. Homozygous males and females were obtained by subsequent crossings. Absence of NRAGE was confirmed by western blot analysis (anti-NRAGE, 1:1000³⁸). All phenotypic analysis was performed on mice that had been backcrossed to the C57BL/6 genetic background for a minimum of six generations. Genotyping was performed by PCR using primers 93 and 76 to detect the knockout allele; and primer 92: 5'-CATGTATCTGGCCAAACC-3' and primer 172 to detect the wild-type allele.

Histopathology and necropsy. Standard phenotyping was carried out at the 'Institut Clinique de la Souris' (ICS, Strasbourg, France), on adult *NRAGE* knockout males with age-matched wild-type controls. The phenotyping battery included macroscopic inspection of organs, organ weights (liver, kidney, spleen, heart, visceral fat), total body weight and histopathological evaluation of organs (integument, cardiovascular, respiratory, immune/hematopoietic, digestive tract, digestive organs, urogenital, musculoskeletal, endocrine system, central nervous system, peripheral nervous system and sensory organs). Analysis of skeletons was performed on newborn mice following standard alcian blue and alizarin red staining.

Cell cycle analysis. Mouse embryonic fibroblasts were isolated from E13.5 *NRAGE* knockout and wild-type littermates. Cells were synchronized to G₀ phase by serum starvation for 48 h, then allow to enter the cell cycle by adding serum to the medium. Analysis of relative cellular DNA content was analyzed using propidium iodide and flow cytometry following standard protocols.

Hair follicle analysis. Expression of *NRAGE* during the first hair follicle cycle was analyzed by *in situ* hybridization and immunoblot on dermal cryosections and lysates from C57BL/6 mice at P0, P2, P4, P8, P12, P17, P18 and P21 following standard protocols. For the immunostaining analysis, P18 back skin samples were immersion fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 4 h, left overnight in 30% PBS-sucrose, and embedded in OCT. Here, 15- μ m thick sections were serially cut and mounted on superfrost[®]/Plus slides (Fisherbrand). Primary antibodies against NRAGE and p75NTR³⁸ were used at a dilution of 1/100 following standard protocols. For the analysis of the first hair follicle cycle, back skins from *NRAGE* knockout and wild-type littermates were harvested at P1-P2, P14, P17 (two p75NTR knockout and two wild-type littermates used as control) and P21. For the anagen–catagen transition after HF cycle induction, 6- to 8-week-old male mice were depilated with a wax and rosin mixture and skin samples were harvested 19 days post depilation. All skin samples were fixed either in 4% PFA or Bouin's fixative, embedded in paraffin and sectioned parallel to the vertebral line to obtain longitudinal HF sections.

Sympathetic neurons analysis

Apoptosis assays. Primary sympathetic neurons were isolated from P6 SCG of *NRAGE* knockout and wild-type mice and cultured as previously described.⁴⁵ Neurons were maintained in 20 ng/ml of NGF (Harlan) for 2 days, then NGF was removed, the cells rinsed and switched to media containing anti-NGF (0.1 μ g/ml, Chemicon International) together with 12.5 mM KCl, to promote survival, with or without the addition of 200 ng/ml of BDNF for 48 h. Then cells were fixed in 4% paraformaldehyde and stained with DAPI (Vector Labs) to score the nuclei as apoptotic or non-apoptotic. Approximately 50–70 neurons were counted for each condition. The experiment was performed in triplicate.

Lentiviral infection. p75NTR and NRAGE knockdowns were achieved by infecting rat sympathetic neurons with lentivirus encoding miRNA with sequences directed against rat *p75NTR* or *NRAGE* mRNA. The miR targeting sequences were designed using the Invitrogen miR prediction algorithm and cloned following the manufacturer's instructions into a variant of the pcDNA6.2/GW-EmGFP-miR vector (Invitrogen) in which the EmGFP has been replaced with mRFP. VSV-G pseudotyped viral particles were produced in 293-T cells and particles were purified by ultracentrifugation, resuspended in DMEM, and the amount of active viral particles was determined by titration on HEK293T cells. For miR-mediated knockdown, sympathetic neurons were transduced at an MOI of 10 and analyzed 5–6 days post-transduction. Apoptosis assays were realized by treating the rat cells with BDNF for 48 and 72 h following the protocol described above. JNK activation was analyzed by stimulating the cell with BDNF (200 ng/ml) for one hour. The cells were then lysed in NP-40 buffer and immunoblot for p-JNK, JNK1, NRAGE, p75NTR and tubulin.

Neuronal counts of SCGs. SCGs from E17, P3 and P23 *NRAGE* knockout and wild-type littermates were immersion fixed overnight in formalin and embedded in paraffin. Here, 6- μ m thick sections were serially cut and mounted on superfrost[®]/Plus slides (Fisherbrand). Slides were stained with cresyl violet, scanned with Miraxscan scanner (Zeiss) and neuronal numbers were determined by counting nucleoli present in neuronal profiles on every third section for P3 and P23 and every sixth for E17.

Developmental motoneuron death. Apoptosis in spinal cords was analyzed by whole-mount immunohistochemistry using an antibody directed against cleaved caspase-3 (Asp175, Cell Signaling Technologies; 1/200) following a previously described protocol.³⁰ For quantification, spinal cords were divided into five regions (cervical, brachial, thoracic, lumbar and sacral) and the number of apoptotic cells was determined in each region. Four *NRAGE* knockout, five wild-type littermates, eight *p75NTR* knockout and 12 wild-type littermates were analyzed.

Nerve outgrowth. Peripheral innervation was visualized at E11.5, E12.5 and E13.5 by whole-mount immunohistochemistry using a monoclonal antibody (TuJ1) recognizing the neuron-specific β -tubulin III protein. A minimum of three knockout and three wild-type littermates were observed for each genotype at all stages of development.

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