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# Homeodomain-Interacting Protein Kinase-2 Regulates Apoptosis in Developing Sensory and Sympathetic Neurons

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## Summary

**Excess neurons in the developing nervous system are eliminated by apoptosis, an ordered cascade of proteolytic events orchestrated by the caspase family of proteases [1]. The apoptotic machinery is tightly regulated by a variety of extracellular signals that either activate or suppress apoptosis after binding to receptors on neurons [2]. These signals are integrated in neurons by a complex network of protein-protein interactions that bring about transcriptional and post-translational changes in key regulators of the apoptotic machinery; such regulators include members of the Bcl-2 family [3]. Homeodomain-interacting protein kinase-2 (HIPK2) is a recently identified nuclear serine-threonine kinase that interacts with homeodomain transcription factors [4, 5] and participates in the regulation of cell growth [6] and genotoxic stress-induced apoptosis [7, 8]. Here we show that overexpression of HIPK2 in developing neurotrophin-dependent sensory and sympathetic neurons promotes apoptosis of these neurons grown with neurotrophins. HIPK2-induced apoptosis is caspase-dependent, is inhibited by overexpression of Bcl-2 and Bcl-W, and fails to occur in Bax-deficient neurons. Trigeminal sensory neurons, which are especially susceptible to HIPK2-induced apoptosis, express the highest levels of HIPK2 during the peak of apoptosis in vivo. Knockdown of endogenous HIPK2 with antisense oligonucleotides substantially reduces and delays apoptosis after neurotrophin deprivation in vitro. These findings identify HIPK2 as a novel participant in programmed cell death in the developing peripheral nervous system.**

## Results and Discussion

### HIPK2 Expression in Developing Sensory and Sympathetic Ganglia

The predominant expression of Homeodomain-interacting protein kinase-2 (HIPK2) in the brain, spinal cord, and retina of fetal mice [9] together with previous work on the on the pro-apoptotic effects of HIPK2 in various cell lines [7, 8] raised the possibility that it may play a role in regulating neuronal apoptosis during development. To determine if HIPK2 is expressed in experimentally tractable populations of neurons, we used competitive RT/PCR to measure the levels of HIPK2 mRNA in three well-characterized groups of peripheral sensory and sympathetic neurons. Trigeminal, nodose, and superior cervical ganglia were dissected from CD1 mouse embryos at stages during the phase of naturally occurring neuronal death. Figure 1 shows that HIPK2 was detectable in all three ganglia. Whereas the levels HIPK2 mRNA relative to mRNA for the housekeeping protein GAPDH were essentially unchanged throughout this period of development in the nodose and superior cervical ganglia (SCG), there was a marked developmental change in the expression of HIPK2 relative to GAPDH mRNA in the trigeminal ganglion. From a peak at E12, the relative level of HIPK2 mRNA falls 3-fold by P1. The peak expression just precedes the peak number of neurons in the trigeminal ganglion at E13, and the decrease in expression occurs throughout the period of naturally occurring neuronal death, which is complete by birth. Over this period of development, about half the number of neurons in the ganglion die by apoptosis [10, 11]. Thus, the levels of HIPK2 in the trigeminal ganglion are high during at the commencement of naturally occurring death and much lower at the end of this phase of development.

To determine whether HIPK2 is expressed in neurons, we used differential sedimentation to separate neurons from neuronal cells in the trigeminal ganglion at E18 and P1, when the size difference between neurons and other cell types is sufficient to allow neuron preparations to obtain greater than 95% purity. Figure 1 shows a clear HIPK2 mRNA signal in these purified neurons, indicating the HIPK2 is present in developing neurons.

### Overexpression of HIPK2 Induces Apoptosis in Sensory and Sympathetic Neurons

We began investigating the potential effect of HIPK2 on neuronal survival by overexpressing this protein in neonatal neurons cultured in the presence of a neurotrophin that promotes their survival (nerve growth factor [NGF] in the case of trigeminal and SCG neurons and brain-derived neurotrophic factor [BDNF] in the case of nodose neurons). Dissociated, purified neurons were plated and grown overnight with the respective neurotrophin and microinjected with either a plasmid expressing HIPK2 or an empty control plasmid. Injected neurons were cultured for an additional 96 hr in the presence of neurotrophin, and the number of surviving neurons was counted 48 and 96 hr after injection. Figure 2 plots the number of surviving neurons at these time points and expresses them as a percentage of the number injected. Whereas virtually all control-injected neurons were still surviving after 96 hr, overexpression of HIPK2 caused highly significant decreases in survival in all three kinds

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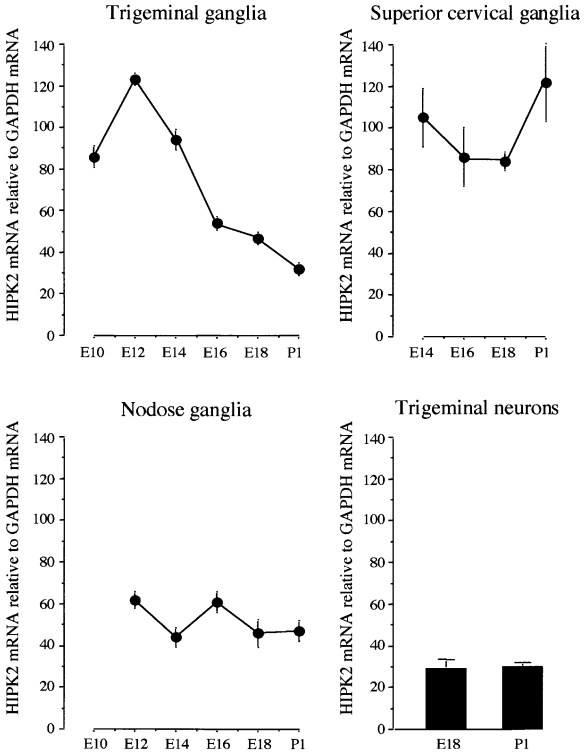


Figure 1. Developmental Changes in the Level of HIPK2 mRNA in Sensory and Sympathetic Ganglia

The level of HIPK2 mRNA relative to GAPDH mRNA was measured at two-day intervals in the trigeminal ganglion from E10 to P1, in the nodose ganglion from E12 to P1, and in the SCG from E14 to P1. The relative levels of HIPK2 mRNA are also shown for purified trigeminal ganglion neurons at E18 and P1. The means and standard errors of four independent measurements at each age are shown.

of neurons at both time points. Similar decreases were observed in BDNF-supplemented nodose neurons and NGF-supplemented SCG neurons (approximately 18% reduction relative to control-injected neurons at 48 hr and between 55% and 64% reduction at 96 hr). However, HIPK2 overexpression was most effective in promoting the death of trigeminal neurons; 73% were already dead by 48 hr, and 94% were dead by 96 hr. These results indicate that HIPK2 is capable of bringing about the death of developing, neurotrophin-dependent sensory and sympathetic neurons.

### HIPK2 Kills Neurons by a Caspase-Dependent, Bax-Dependent Mechanism

We began investigating the mechanism by which HIPK2 overexpression promotes neuronal death by determining whether it depends on caspases, the proteases that dismantle the cell in programmed cell death. Because of the pronounced effect of HIPK2 overexpression on trigeminal neurons, we focused on these neurons. Figure 3 shows that the pancaspase inhibitor Z-VAD-FMK largely prevented HIPK2-induced death of trigeminal neurons: there was no significant difference in the number of Z-VAD-FMK-treated HIPK2-overexpressing neurons and control-injected neurons by 48 hr, and by 96 hr only 27% of the HIPK2-overexpressing neurons had died in Z-VAD-FMK-treated, cultures compared with untreated neurons. These results indicate that HIPK2-induced neuronal death is a caspase-dependent process.

Because members of the Bcl-2 family are key regulators of caspase activation [3], we investigated whether manipulating the expression of pro-apoptotic and anti-apoptotic members of this family could rescue trigeminal neurons from HIPK2-induced cell death. Among the proapoptotic Bcl-2 proteins, Bax plays a key role in regulating the survival of sensory and sympathetic neurons. Targeted deletion of Bax largely prevents naturally occurring neuronal death of these neurons in vivo and substantially reduces apoptosis after neurotrophin withdrawal in vitro [12, 13]. We therefore established cultures from Bax-deficient neonates to see if Bax is required for HIPK2-induced cell death. Figure 3 shows that Bax-deleted neurons were almost completely protected from HIPK2-induced neuronal death, indicating HIPK2-induced apoptosis requires Bax.

Anti-apoptotic members of the Bcl-2 family prevent apoptosis in part by dimerizing with and inhibiting the function of pro-apoptotic members of this family. Overexpression of the anti-apoptotic proteins such as Bcl-2 and Bcl-W in developing sensory and sympathetic neurons can rescue a substantial number after neurotrophin

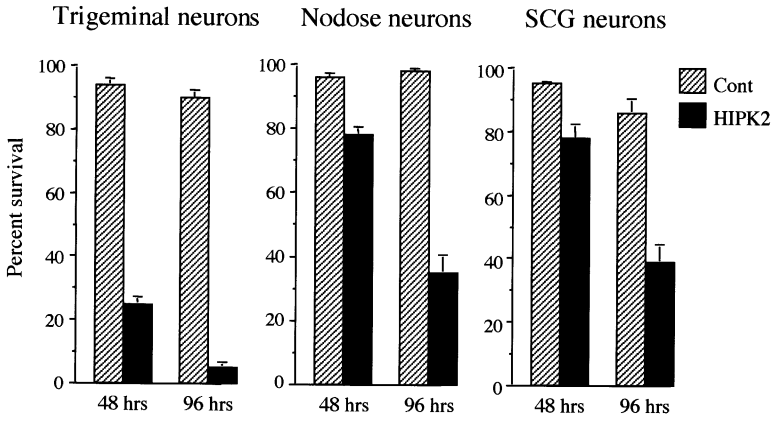


Figure 2. HIPK2 Overexpression Kills Sensory and Sympathetic Neurons Grown with Neurotrophins

Cultures of newborn mouse trigeminal, nodose, and SCG neurons were grown overnight with either 20 ng/ml NGF (trigeminal and SCG neurons) or 20 ng/ml BDNF (nodose neurons), then injected with either the HIPK2 expression plasmid or an empty control plasmid (pcDNA3.1 without an insert) and incubated for an additional 96 hr with 5 ng/ml of the same neurotrophin. The numbers of surviving neurons were counted at 48 and 96 hr after injection and expressed as a percentage of the number injected. The means and standard errors of the results of four separate experiments are shown. In each experiment, 80–150 neurons were injected for each experimental condition.

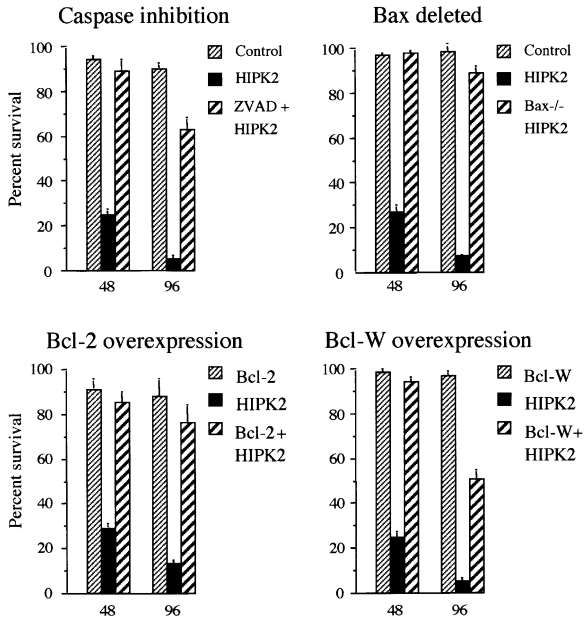


Figure 3. Inhibition of HIPK2-Induced Apoptosis by Caspase Inhibition and Bcl-2 Proteins

Cultures of newborn mouse trigeminal neurons were grown overnight with 20 ng/ml NGF, then injected with plasmids expressing HIPK2, Bcl-2, or Bcl-W alone or in combination, as indicated. For the caspase inhibition experiments, the Z-VAD-FMK pancaspase inhibitor was added to cultures at a concentration of 75 $\mu$M immediately after injection. The number of surviving neurons was counted after an additional 48 and 96 hr incubation in medium containing 5 ng/ml NGF and expressed as a percentage of the number injected. To study the effect of the absence of Bax on HIPK2-induced apoptosis, cultures were established from $Bax^{-/-}$ mice ($Bax^{-/-}$ + HIPK2) and wild-type littermates (control and HIPK2). The means and standard errors of the results of four separate experiments are shown. In each experiment, 80–150 neurons were injected for each experimental condition.

withdrawal [14–16]. For this reason, we also investigated whether overexpressing Bcl-2 and Bcl-W could prevent HIPK2-induced neuronal death. As shown in Figure 3, overexpression of either Bcl-2 or Bcl-W largely inhibited the HIPK2-induced apoptosis, Bcl-2 being somewhat more effective than Bcl-W. Overexpression of the anti-apoptotic Bcl-XL protein also markedly inhibited the death of HIPK2-transfected trigeminal neurons, although not as effectively as Bcl-2 and Bcl-W (data not shown).

### Endogenous HIPK2 Facilitates Apoptosis of NGF-Deprived Neurons

The demonstration that overexpression of HIPK2 causes Bax-dependent apoptosis in neurons grown with NGF raised the possibility that HIPK2 normally plays a role in programmed cell death in the developing nervous system. To investigate this possibility directly, we injected antisense HIPK2 oligonucleotides into cultured neurons to reduce expression of endogenous HIPK2 protein and see if this would prevent neuronal death after NGF deprivation. In these experiments, sympathetic and trigeminal neurons were grown with NGF overnight and injected the following morning with antisense HIPK2 or a control oligonucleotide and subsequently grown without NGF. After NGF deprivation, control-injected sympathetic neurons died very rapidly (Figure 4A), and control-injected trigeminal neurons died more slowly (Figure 4B). These losses were very similar to those observed in uninjected, NGF-deprived sympathetic and sensory neurons, respectively (data not shown). In marked contrast, antisense-injected neurons died much more slowly. Whereas virtually all control-injected sympathetic neurons were dead 48 hr after NGF deprivation, 35% of antisense-injected neurons were still surviving at this time (Figure 4A). Whereas 59% of control-injected trigeminal neurons were dead 48 hr after NGF deprivation, only 18% of antisense-injected neurons had died at this time, and by 96 hr, the loss of antisense-injected neurons was only 41%, compared with 75% of control neurons (Figure 4B). To confirm that the antisense HIPK2 oligonucleotides reduce HIPK2 expression, we transfected CHO-K1 cells with these oligonucleotides and assessed the level of HIPK2 mRNA 15 hr later by RT-PCR. Figure 4C shows a clear reduction in the HIPK2 signal in antisense-transfected cells compared with control transfected cells. Taken together, these results suggest that HIPK2 plays an important role in bringing about the death of NGF-dependent neurons deprived of NGF. Interestingly, the cell bodies of the antisense HIPK2-transfected neurons surviving without NGF were much smaller than those of neurons grown with NGF (not shown). This appearance is reminiscent of the atrophic cell bodies of Bax-deficient neurons surviving in culture without NGF [12].

Because of the very rapid death of P1 sympathetic neurons after NGF deprivation and the especially clear effect of HIPK2 knockdown in rescuing these neurons, we investigated whether NGF deprivation might affect expression of HIPK2 mRNA in these neurons. For these experiments the neurons were grown with and without NGF for 48 hr, and RT/PCR was used to assess the relative levels of HIPK2 mRNA. To maintain similar numbers of neurons under both experimental conditions, the cultures were supplemented with the pancaspase inhibitor Z-VAD-FMK to prevent cell death. After 48 hr incubation, the level of HIPK2 mRNA was significantly elevated 2.17- $\pm$ 0.07-fold in NGF-deprived neurons compared with NGF-treated neurons ($p < 0.003$, t test, $n = 4$ independent experiments). This suggests that HIPK2 function is regulated at least in part at a transcriptional level in these neurons after NGF deprivation.

### Conclusions

We have identified HIPK2 as an important participant in programmed cell death in the developing peripheral nervous system. HIPK2 is expressed in sensory and sympathetic neurons during the stage of programmed cell death in vivo. Overexpression of HIPK2 kills these neurons by a caspase-dependent mechanism in the presence of neurotrophins, and knockdown of endogenous HIPK2 inhibits apoptosis after neurotrophin withdrawal in vitro. These findings suggest that HIPK2 plays a role in bringing about apoptosis in neurons that fail to obtain an adequate supply of neurotrophic factors in vivo. HIPK2 has previously been implicated in regulating apoptosis in various cell lines by several different

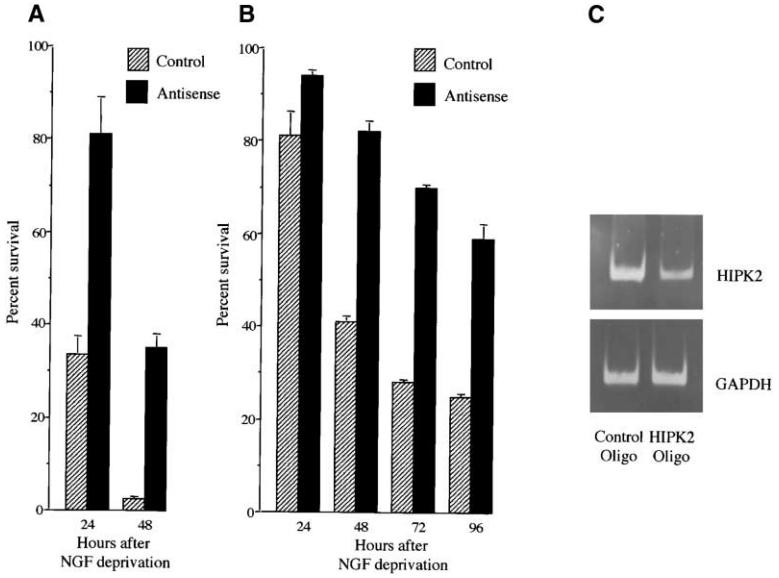


Figure 4. Endogenous HIPK2 Facilitates Apoptosis of NGF-Deprived Neurons

Cultures of newborn mouse SCG neurons (A) and trigeminal neurons (B) were grown overnight with 20 ng/ml NGF, then injected with either antisense HIPK2 or control oligonucleotides. They were then grown in medium without NGF. The number of surviving neurons was counted at 24-hourly intervals and expressed as a percentage of the number of injected neurons. The means and standard errors of the results of four separate experiments are shown. In each experiment, 80–150 neurons were injected for each experimental condition. (C) Gels showing HIPK2 and GAPDH PCR products derived from reverse-transcribed RNA obtained from CHO-K1 cells 15 hr after transfection with either antisense HIPK2 or control oligonucleotides. The levels of GAPDH are very similar in antisense HIPK2 and control transfected cells, whereas the level of HIPK2 is very clearly reduced in the antisense-transfected cells.

mechanisms. In response to genotoxic stress, HIPK2 promotes p53-mediated apoptosis by phosphorylating p53 [7, 8] and by inhibiting the p53 negative regulator MDM2 [17]. HIPK2 phosphorylates the transcriptional corepressor CtBP, resulting in proteosomal degradation of CtBP and induction of proapoptotic genes independently of p53 [18]. HIPK2 also positively regulates TGF-β-induced JNK activation and apoptosis independently of p53 [19]. Although the mechanisms by which HIPK2 brings about apoptosis in developing neurons remain to be ascertained, it should be noted that p53 and JNK have been implicated in sympathetic neuron apoptosis after NGF deprivation [20, 21]. In addition to defining the transcriptional and posttranslational events that mediate the proapoptotic effects of HIPK2 in developing neurons, it will be important in future work to ascertain whether extracellular signals that regulate neuronal survival and death influence the expression and/or kinase activity of HIPK2. In summary, our work has revealed a novel level of control in the apoptotic machinery of developing neurons.

## Experimental Procedures

### Neuron Cultures and Microinjection

Dissociated cultures of neurons from the trigeminal, nodose, and SCG of newborn or postnatal-day (P1) mice were set up as described previously [22] except that the neurons were purified free of nonneuronal cells via a percoll gradient (Amersham) and were grown in neurobasal medium with B-27 supplement and glutamax (Invitrogen). After cultures were incubated overnight with either 20 ng/ml NGF (trigeminal and SCG) or 20 ng/ml BDNF (nodose), intranuclear injection of expression plasmids or antisense oligonucleotides was carried out [15]. Plasmid DNA, prepared with the QIAfilter midiprep kit (Qiagen), and oligonucleotides (Proligo) were injected in phospho-buffered saline (PBS) (pH 7.4) at final concentrations of 0.15 μg/ml and 2 μg/ml, respectively. The HIPK2 antisense oligonucleotide was an LNA-DNA-LNA gapmer with the following sequence: 5′-gggCCACCGCGGCCATggat-3′. The control was an LNA-DNA-LNA gapmer with no homology to any known sequence: 5′-cctaGC GTGACGCtatc-3′. A fluorescent dextran dye, Alexa Fluor (Molecular Probes, Eugene, OR), was coinjected (final concentration 0.35 mg/ml) with the plasmid or oligonucleotide so that microinjected neurons could be distinguished from non-microinjected neurons. After injection, the neurons were grown in medium containing 5 ng/ml of the same neurotrophins unless otherwise stated. The number of surviving neurons was counted at 24- or 48-hourly intervals after injection and is expressed as a percentage of the number of injected neurons. Around 100 neurons were injected for each experimental condition in each experiment.

### Measurement of HIPK2 mRNA

A semiquantitative RT/PCR assay determined the expression pattern of HIPK2 mRNA in dissected ganglia, purified neurons, and cultured neurons. Total RNA was purified with the nucleospin RNA II kit (Macherey-Nagel) and recovered in 100–1,000 μl (depending on the tissue size) of DEPC-treated $H_2O$. The RNA was reverse transcribed for 1 hr at 37°C with superscript enzyme (Invitrogen) in a 30 μl reaction containing the manufacturers' buffer supplemented with 0.5 mM dNTPs (MBI Fermentas) and 10 μM random hexanucleotides (Amersham). A 3 μl aliquot of each reverse transcription reaction was amplified in a 30 μl PCR reaction containing 1 × PC2 buffer (Helena BioSciences), 0.1 mM dNTPs, 0.85 units of Taq Supreme (Helena BioSciences), and 20 pmol primers. The forward and reverse primers for HIPK2 were 5′-GCCTACCTTACGAGCAGACC-3′ and 5′-CGACGCATTAGGTTATGTGG-3′, respectively. These primers hybridize 121 bp apart in the mouse HIPK2 sequence. HIPK2 cDNA was amplified by cycles at 95°C for 50 s, followed by 50 s at 53°C, followed by 50 s at 68°C. The reaction was completed with a 10 min extension at 68°C. The reproducibility of the HIPK2 results were ascertained by repetition of each PCR twice for two different (27 and 29) numbers of cycles. The cycling conditions were optimal for the amplification of the HIPK2 transcript so that the rate of reaction did not plateau. Quantitative, competitive RT/PCR was also used for determining the amount of mRNA for the housekeeping GAPDH protein in each RNA sample [15]. The PCR products were separated on 8% nondenaturing polyacrylamide gels. These gels were subsequently stained with SyberGold (Cambridge Biosciences), and the intensity of the RT-PCR products was determined via a gel documentation system (Biogene) with Phoretix software (Phoretix International).

### Validation of HIPK2 Knockdown by Antisense Oligonucleotides

CHO-K1 cells were transfected with the antisense and control LNA-DNA-LNA gapmers with Optimem and Oligofectamine in accordance with the manufacturer's instructions (Invitrogen). After 15 hr, RNA purification and RT-PCR were carried out as described above. The forward primers for HIPK2 and GAPDH were 5′-TGGTCAAGTG

CTGGAAC-3′ and 5′-CTTGTCATCAACGGGAAG-3′, respectively. The reverse primers for HIPK2 and GAPDH were 5′-ACTTCAAT CTGGCCTTGC-3′ and 5′-CAACATACTCGGCACCAG-3′, respectively. These primers hybridize 94 bp and 88 bp apart in the hamster HIPK2 and GAPDH sequences.

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