mRNA for N-Bak, a neuron-specific BH3-only splice isoform of Bak, escapes nonsense-mediated decay and is translationally repressed in the neurons

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mRNA for neuronal Bak (N-Bak), a splice variant of pro-apoptotic Bcl-2 family member Bak is expressed in the neurons. Surprisingly the endogenous Bak protein cannot be demonstrated in the neurons, although the antibodies recognize N-Bak protein from in vitro translation or transiently transfected cells. As N-Bak mRNA contains premature termination codon (PTC) at 89 nucleotides upstream from the last exon–exon junction, it could be degraded by nonsense-mediated decay (NMD) during the pioneer round of translation thus explaining the absence of the protein. We show here that the endogenous neuronal N-Bak mRNA is not the NMD substrate, as it is not accumulating by cycloheximide treatment, it has a long lifetime, and even prevention of PTC by interfering with the alternative splicing did not lead to translation of the Bak mRNA. N-Bak protein is also not revealed by proteasome inhibitors. Our data suggest strong translational arrest of N-Bak mRNA in the neurons. We show that this arrest is partially mediated by 5'-untranslated region of Bak mRNA and it is not released during mitochondrial apoptosis.

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Nonsense-mediated mRNA decay (NMD) is a cellular surveillance mechanism to recognize and degrade mRNAs harboring premature translation-termination codon (PTC). Truncated proteins encoded by such mRNAs could be deleterious for the cell. During pioneer round of translation, the cell carries out quality control of the mRNA and either passes it to produce the proteins or directs it to the NMD-mediated degradation.1,2 Specific multiprotein exon junction complexes deposited on the exon–exon junctions during pre-mRNA splicing are detected and removed by the ribosome during pioneer round of translation. In mammals, one of the main criteria for the NMD substrate mRNAs is that distance of PTC by interfering with the alternative splicing did not lead to translation of the Bak mRNA. We show here that N-Bak mRNA is not the NMD substrate in the neurons and thus belongs to mRNAs that escape the NMD despite the correspondence to the 55-nt NMD rule. Our data suggest that N-Bak mRNA is degraded in the neurons by NMD that causes translationally arrested and its 5'-untranslated region (UTR) is partially responsible for this arrest.

Results

N-Bak mRNA but not protein is expressed in the neurons. Earlier we6 and others9 were not able to detect N-Bak mRNA in the neurons (although Uo et al8 reported the protein by immunoblot). The PTC on the N-Bak mRNA locates at 89 nucleotides upstream from the last exon–exon junction (Figure 1c) corresponding to the 55-nt NMD rule. Thus, the N-Bak mRNA could be degraded during the pioneer round of translation, explaining the absence of the protein.

In this study we set up to test the hypothesis that N-Bak mRNA is degraded in the neurons by NMD that causes absence of the protein. Our results suggest that N-Bak mRNA is not the NMD substrate in the neurons and thus belongs to the mRNAs that escape the NMD despite the correspondence to the 55-nt NMD rule. Our data suggest that N-Bak mRNA is translationally arrested and its 5'-untranslated region (UTR) is partially responsible for this arrest.

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Keywords: N-Bak; nonsense-mediated decay; alternative splicing; mRNA; cortical neurons

Abbreviations: NMD, nonsense-mediated decay; qRT-PCR, quantitative real-time PCR; PTC, premature termination codon; CHX, cycloheximide; ActD, actinomycin D; UTR, untranslated region

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We first tested the antibodies on the in vitro translated N-Bak and Bak and on the HeLa cells transiently transfected with the respective expression plasmids. Two antibodies (Sigma B-5897 and EMD Millipore 06-536) against the N-terminal region common for both Bak and N-Bak, whereas the epitopes for two latter antibodies are not precisely defined.

We then compared the levels of transcripts and proteins of Bak isoforms in the mouse brain. The cultured cells used in this study were analyzed as well. Transcripts for Bak isoforms were detected by RT-PCR with primers flanking exon N6 (Figure 1c), thus amplifying both mRNAs with comparable efficiency. As shown on Figure 1a, in the newborn (P0) mouse brain the level of N-Bak transcripts (from the neurons) is highly surpassing the level of BH1-3 Bak transcripts (from the non-neuronal cells) that is in agreement with the results of RNase protection assay.6 N-Bak transcripts were easily detected in the cultures of cortical and sympathetic neurons; the low levels of Bak transcripts in these cultures result from the few contaminating non-neuronal cells.6 Rat pheochromocytoma PC6 cells express exceptionally the mRNAs for both Bak isoforms, the levels of N-Bak mRNA being very low. As expected, only Bak but not N-Bak transcripts were detected in the P0 mouse liver tissue and NIH-3T3 mouse fibroblast cells. The proteins were analyzed from the other half of the same samples by immunoblot. In agreement with previous study6 the anti-Bak antibody (EMD Millipore) did not detect N-Bak protein in any of the tissues or cells whereas the Bak protein was always detected (Figure 1b). The same results were obtained with antibody from Sigma, whereas other two antibodies gave again poor results (data not shown).

Thus, despite the high levels of N-Bak mRNA the protein was not detected in the neurons, although our assay easily revealed Bak protein from the samples with significantly lower Bak mRNA levels. We conclude that the absence of N-Bak protein in the neurons results from the posttranscriptional regulation rather than minute amounts of the mRNA. We considered three mechanisms that could lead to the absence of N-Bak protein: (i) rapid degradation of the mRNA, (ii) rapid degradation of the protein and (iii) translational block of the mRNA.

N-Bak mRNA is not the substrate for NMD. Inclusion of exon N to the Bak mRNA generates PTC that corresponds to the 55 nt NMD rule (Figure 1c). Such mRNA could potentially be degraded during the pioneer round of translation thus explaining the absence of N-Bak protein. To test this hypothesis we blocked translation by cycloheximide (CHX) that should lead to rapid accumulation of the NMD substrate mRNAs.10,11 We treated cultured cortical neurons with CHX for 4 and 8 h and determined the levels of N-Bak transcripts by quantitative RT-PCR (qRT-PCR). mRNA for Bax, another pro-apoptotic Bcl-2 family member that does not contain PTC was also analyzed for comparison. The data were normalized to two reference mRNAs – neurofilament, medium polypeptide (NEFM) and enolase 2 (ENO2) that, just as N-Bak, are specifically expressed in the neurons but not in the small number of non-neuronal cells that are always present in the cultures. The fold changes were calculated in relation to samples treated with vehicle (DMSO) using relative expression software tool REST.12 As shown in Table 1, blockage of translation for 4 h did not lead to accumulation of N-Bak or Bax transcripts in the cortical neurons, whereas of the four anti-Bak antibodies results in high non-specific background (data not shown). Thus, at least two antibodies specifically detected N-Bak protein when it is expressed from the plasmid.
small but significant accumulation of N-Bak but not Bax occurs by 8 h of CHX treatment. However, this increase was completely blocked by transcription inhibitor actinomycin D (ActD). Thus, this late and small increase in the N-Bak mRNA was caused by increased transcription, most probably because of CHX-induced cellular stress. The same results were obtained with another pair of N-Bak-specific primers (mouse N-Bak-2, data not shown), or when data were calculated in relation to untreated samples instead of DMSO-treated samples (data not shown). In the PC6 cells, similar CHX-induced increase in the N-Bak mRNA level (normalized to beta actin) was observed by 4 h of treatment but was also completely blocked by co-treatment with ActD (data not shown).

As a rule, the NMD substrate mRNA levels are low because of ongoing degradation. If N-Bak mRNA were a substrate for NMD in the neurons, its turnover should be extremely rapid, as the levels of this transcript in the brain and cultured neurons are high (Figure 1). To study the stability of N-Bak mRNA we blocked transcription in the cortical cultures by ActD and determined the time-dependency of N-Bak and Bax mRNA degradation by qRT-PCR. The data were normalized to 7SL RNA (RN7SL1) and 18s ribosomal RNA (Rn18s) that are transcribed by RNA polymerase III and thus not blocked by ActD. As shown on Figure 2, the N-Bak transcripts were stable in the cortical neurons, whereas the levels of Bax transcripts decreased in time (Figure 2b). Longer treatments with ActD caused general deterioration of the cortical cultures that makes the analysis unreliable. Similar results were obtained using another pair of N-Bak-specific primers (mouse N-Bak-2, data not shown). In the ActD-treated PC6 cells, the transcripts of N-Bak decreased with the same rate as the transcripts of Bax (data not shown).

In summary, the N-Bak mRNA is remarkably stable in the neurons. Such stability is not compatible with the NMD substrate transcripts. Thus, our data do not support the hypothesis that N-Bak mRNA is degraded in the neurons by NMD.

Table 1 Relative changes of N-Bak and Bax transcripts in the cortical neurons treated with CHX or co-treated with cycloheximide (CHX) and actinomycin D (ActD) as determined by qRT-PCR and analyzed by relative expression software tool REST

| Transcript Type | CHX (n = 3) | CHX+ActD (n = 4) |
|-----------------|------------|-----------------|
| Eno2 REF        | 0.9019     | 0.8876          |
| NEFM REF        | 0.8904     | 0.8904          |
| N-Bak TRT       | 0.8881     | 0.9314          |
| Bax TRT         | 0.81       | 0.9589          |

Abbreviations: ENO2, enolase 2; NEFM, neurofilament, medium polypeptide. The data of the target transcripts (TRT) are normalized to the indicated reference transcripts (REF). The sample groups (CHX or CHX+ActD) are compared with control groups (DMSO). P(H1) designates the probability of alternate hypothesis that difference between sample and control groups is due only to chance n designates the number of independent repeat experiments.

Figure 2 Stability of N-Bak mRNA. Cortical neurons were treated with the translation inhibitor ActD (5 μg/ml) and analyzed by qRT-PCR at indicated time points. The levels of N-Bak and Bax transcripts were normalized to 75S RNA (RN7SL1) and Rn18s. Each time point was calculated in relation to untreated samples by relative expression software tool REST. The trends are shown by black lines. The data are results of three independent experiments.

Prevention of alternative splicing and PTC did not lead to translation of N-Bak mRNA. The experiments with CHX and ActD treatment described above suggest that despite of the PTC falling under the 55-nt NMD rule, N-Bak mRNA is not the NMD substrate in the neurons. To study the issue further we tested whether this mRNA could be translated without PTC. To that end we designed Vivo-Morpholino oligonucleotides against exon N that should prevent its inclusion into the endogeneous mRNA. Application of the exon N-specific oligonucleotides to the culture medium of cortical neurons for 24 h indeed leads to significant appearance of Bak mRNA with concomitant disappearance of N-Bak mRNA, whereas control oligonucleotides had no effect (Figure 3a). However, immunoblot with anti-Bak antibody (06-536, EMD Millipore) did not reveal upregulation of Bak protein in such cortical cultures forced to express Bak mRNA (Figure 3b). Addition of
Figure 3 Bak mRNA is not translated in the absence of PTC. (a) RT-PCR analysis of the transcripts of Bak (510 nt) and N-Bak (530 nt) in the cultured cortical neurons treated with exon N-targeting or control Vivo-Morpholino oligonucleotides at 4 or 8 μg/ml for 24 h. N-Bak mRNA is converted into Bak mRNA with exon N-specific (target oligos) but not control oligos. Qualitatively same results were obtained in three independent experiments. (b) Upper panel: immunoblot with Bak-specific antibodies (06-536, EMD Millipore) from the other half of the same samples as in (a). The position of about 25-kD Bak is shown on the lane of HeLa cells express Bak-L76E. The Bak on the neuronal lanes comes from the contaminating glial cells and its level is not increased by the oligonucleotide treatment. Lower panel: reprobing the same filter with antibodies to β-actin. Qualitatively same results were obtained in three independent experiments.

Figure 4 N-Bak protein is not revealed by proteasome inhibition. Cortical neurons were treated with proteasome inhibitor MG-132 for 16 or 24 h in the presence or absence of caspase inhibitor BAF, or with the vehicle (DMSO). Upper panel shows the immunoblot with anti-Bak antibodies (06-536, EMD Millipore). Shown are the samples from HeLa cells transiently transfected with the inactive L76E mutants of Bak and N-Bak. Lower panel shows the same filter reprobed with anti-Hsp70 antibodies. Proteasome inhibition did not reveal N-Bak protein in the cortical neurons, although the positive control Hsp70 was strongly induced. The HeLa cells express Hsp70 constitutively. The same results were obtained in three independent experiments.

Figure 5 N-Bak protein is not induced in the apoptotic neurons. Cortical neurons were treated with etoposide for 24 or 36 h in the presence of proteasome inhibitor MG-132 or caspase inhibitor BAF. Untreated and vehicle (DMSO)-treated cultures as well as HeLa cells overexpressing apoptotically inactive L76E mutants of N-Bak and Bak were included as controls. The immunoblot is probed with anti-Bak antibody (06-536, EMD Millipore). The same results were obtained in three independent experiments.

caspase inhibitor BAF or proteasome inhibitor MG-132 to the culture medium still did not lead to increase in the Bak protein levels in the oligonucleotide-treated cultures (data not shown). Thus, despite the absence of exon N and PTC the endogenous Bak mRNA was not translated in the neurons. The data suggest that a translational arrest rather than NMD causes the absence of N-Bak protein.

**N-Bak protein is not rapidly degraded by proteasome.** One explanation for the absence of N-Bak protein could be its rapid proteasomal degradation. In support of this possibility, the Mobyle program predicted a PEST sequence (signal for proteasome-mediated degradation) encompassing residues 12–33 of mouse Bak/N-Bak. To address this alternative we treated the cortical neurons with proteasome inhibitor MG-132 in the presence or absence of caspase inhibitor BAF. N-Bak protein was not detectable in these conditions whereas control heat shock protein (Hsp70) was strongly upregulated by proteasome inhibition (Figure 4), as published. Thus we did not find evidences for rapid proteasome-mediated degradation of N-Bak protein. Moreover, the BH1-3 Bak protein is easily detected in many cells and tissues despite the presence of this predicted PEST sequence.

**N-Bak protein was not induced in the apoptotic neurons.** As a typical BH3-only protein, N-Bak could function in the Bak-dependent mitochondrial apoptosis. If so, the translational arrest could be released and N-Bak protein expressed in the apoptotic neurons. We treated the cortical neurons with etoposide, the inhibitor of topomerase II that triggers mitochondrial apoptosis, for 24–36 h. The death of the neurons was blocked by caspase inhibitor BAF with or without proteasome inhibitor. Immunoblot with anti-Bak antibodies (06-536, EMD Millipore) (Figure 5) or Sigma (data not shown) again did not show appearance of N-Bak protein in any of these conditions. The same result was obtained when the sympathetic neurons were deprived of nerve growth factor, or treated with etoposide for three days in the presence of BAF (data not shown). Thus, N-Bak mRNA is not translated during the mitochondrial apoptosis, at least not at the detectable level. The membranes were reprobed with antibody Ab-2 used by others to demonstrate N-Bak in the camptothecin-treated cortical cultures but no specific binding was detected (data not shown).

**Translational arrest of N-Bak mRNA is partially mediated by 5′-UTR.** To study the mechanism of translational arrest of N-Bak mRNA we focused on its 5′-UTR that contains two open reading frames upstream of the translation initiation codon. Such 5′-UTRs can participate in the translational repression of mRNAs. We tested the effect of Bak 5′-UTR on the translation using Dual Luciferase Reporter assay with Firefly and Renilla luciferase (FF-Luc and Rn-Luc, respectively). The 5′-UTR of mouse Bak mRNA was cloned upstream of the Firefly luciferase reporter gene, resulting in a Bak-5′-UTR-FF-Luc construct, whereas the reporter without the Bak 5′-UTR (FF-Luc) was used for a reference.
Sympathetic neurons were microinjected with the plasmid mixtures of either Bak-5'-UTR-FF-Luc/Rn-Luc or FF-Luc/Rn-Luc. Relative luciferase activity was determined 48 h later by normalization of FF-Luc activity to Rn-Luc activity. The activity of luciferase in the Bak-5'-UTR-FF-Luc-injected neurons constituted 37.6 ± 3.6% (S.E.M., n = 3) of that in the FF-Luc-injected neurons after 48 h. Thus, the presence of Bak 5'-UTR in front of the FF-Luc gene strongly reduced its activity.

**Discussion**

The main aim of this study was to address whether the N-Bak mRNA is the substrate for NMD that could explain the absence of the encoded protein in the neurons. Inclusion of a 20-nucleotide exon N to N-Bak mRNA causes a translational frameshift and PTC that corresponds to the 55-nt rule of NMD. Such mRNAs should be degraded in the pioneer round of translation and could therefore not produce significant amount of the protein.

The main finding of this study is that N-Bak mRNA is not the substrate for NMD in the neurons. The evidences for this conclusion are the following. (i) Blockage of translation with CHX did not lead to rapid and massive accumulation of N-Bak mRNA in the cortical neurons. (ii) mRNA for N-Bak was remarkably stable in the neurons that is not compatible with translation-associated rapid degradation. (iii) Abrogation of PTC from the neuronal N-Bak mRNA did not lead to its translation. We conclude that neurons have specific mechanism(s) to avoid NMD of the N-Bak mRNA, possibly via translational repression. Of note, we studied here the endogenous PTC-containing transcripts in their natural neuronal environment instead of tumor cell lines transfected with artificial plasmid constructs that might not be subjected to all cellular regulations. On the other hand, blockage of endogenous NMD by siRNAs to Upf1/RENT15 that is a common approach to identify NMD substrates appeared technically too complicated in the primary neurons.

Endogenous N-Bak protein has been detected by others by antibody Ab-2.8 However, different biochemical approaches including various solubilization buffers, immunoprecipitations and so on never revealed endogenous N-Bak protein with any anti-Ak antibody tested, although plasmid-expressed N-Bak was always seen.6

Classically, the NMD has been described for mRNAs where the PTC is generated by aberrations. However, it is estimated that about one-third of alternatively spliced mRNAs have PTC-containing isoform(s) that could potentially be degraded by NMD.4,5 Such physiological generation of PTC could be a mechanism to regulate gene expression, a process termed regulated unproductive splicing and translation (RUST).19 Although the issue is not clear, some studies estimated that only up to 10% of such transcripts accumulate when NMD is blocked15,20,21 showing that a considerable portion can escape the NMD. Our data suggest that N-Bak mRNA is not the typical case of RUST, where the alternative splicing-generated PTC is exploited to downregulate the mRNA.22–24 Instead, the mRNA appears to be maintained in a translationally repressed state independently (and despite) of the PTC (Figure 3). N-Bak mRNA thus belongs to the class of NMD-escape mRNAs which are translationally repressed.25

The mechanisms of translational arrest and the strong stability of N-Bak mRNA remain to be studied. Our results show that the 5'-UTR of Bak mRNA strongly reduces the translation of the reporter gene in the neurons, suggesting that it participates in the translational repression of N-Bak mRNA. 5'-UTR of mouse, rat and human Bak contain short open reading frames upstream of the translation initiation site shown to be essential in the translational repression of the mRNAs.17,18 However, the 5'UTR of Bak did not repress expression of the reporter gene completely, suggesting the participation of other mechanism as well. The potential candidates are the microRNAs known to repress mRNA translation via 3'-UTR.26–29

The splicing of Bak pre-mRNA (inclusion of exon N) is triggered around the time the neuronal progenitors exit the cell cycle (our unpublished data). Moreover, the sequence, regulatory elements (branch point and poly-pyrimidine tract) and the position of exon N between constitutive exons 4 and 5 of the Bak gene are evolutionally conserved. Thus, the absence of Bak (and presence of N-Bak mRNA) is a pan-neuronal feature that seems to last throughout the lifetime of the neurons. It is tempting to speculate that the biological meaning of neuronal Bak pre-mRNA splicing is to get rid of an apoptotic effector Bak. Indeed, the death machinery is under stringent brakes in the postmitotic neurons, such as blockage of Bax protein,30 strong association of the caspases with XIAP protein,31–33 low levels of Apaf-1 (Wright et al.34, Wright et al.35) and so on. Removal of Bak by alternative splicing could be one additional brake to make the neurons more resistant to accidental apoptosis. The biological meaning of N-Bak mRNA remains currently unclear. Exon N is inserted to the Bak pre-mRNA with exact precision (just before the BH1 domain-encoding sequence) that converts the BH1-3-type protein into the BH3-only protein. Thus, the neurons seem to require an additional member to their repertoire of BH3-only proteins and generate it by alternative splicing of Bak. Curiously, the N-Bak mRNA is stabilized and translationally repressed in the neurons, and this repression is not relieved in the classical apoptotic situations (this study) or in many other stress conditions (our unpublished data). When (if ever) this mRNA is translated remains currently unknown.

**Materials and Methods**

**Cell cultures.** The cortical neurons from the embryonic day 15–16 mice, dissociated as published,6 were grown on the polyornithine-coated 35-mm plastic dishes (Becton Dickinson, Franklin Lakes, NJ, USA) in the Neurobasal medium (Invitrogen, Carlsbad, CA, USA) containing 2% of B27 serum supplement and 0.5 mM L-Glutamine and 100 μg/ml Primocin (InvivoGen, San Diego, CA, USA) for 5–6 days before the experiments. The cultures containing many non-neuronal cells were excluded, although completely glia-free cultures were not possible. The experiments were repeated on the independent cultures 3–5 times. The newborn mouse sympathetic neurons were cultured on the dishes coated with polyornithine-laminin (Sigma) in the Neurobasal medium containing B27 supplement (Invitrogen) and 30 ng/ml mouse 2.5 S NGF (Promega, Madison, WI, USA), as published.36,37 PC6 cells were grown in DMEM (Invitrogen) containing...
10% of horse serum (PAAS Laboratories, Pasching, Austria), 5% of fetal bovine serum (HyClone, Thermo Scientific, Loughborough, UK) and 100 μg/ml of Normocin (InvivoGen); HeLa, primary glia and NIH-3T3 cells were grown in DMEM (Invitrogen) containing 10% fetal bovine serum (HyClone) and 100 μg/ml Normocin. The following additives were used: CHX (Sigma) in DMSO at 20 μg/ml, ActD (Sigma) at 5 μg/ml, proteasome inhibitor MG-132 (Sigma) at 10 μg/ml, pan-caspase inhibitor boc-asparil-(OMe)-fluoromethylketone (Baf) (Calbiochem/EMD Biosciences, Darmsstadt, Germany) at 25 μg/ml, etoposide (Sigma) at 4 μg/ml. The cell membrane-penetrating Vivo-Morpholino oligonucleotides (GeneTools, LLC, Philomath, OR, USA) were designed against the region encompassing exon N (5′-ACCTGTCATGGCTGCTGCTC-3′), control sequence (5′-AGCCGACGA TCAATGTCAGACCGG-3′) and applied to the Primeer Express v2.0. software (Applied Biosystems, Carlsbad, CA, USA) or OligoAnalyzer software (eudna.com) and ordered from Sigma. PCR primers were designed as published.6,36,38 FF-Luc. The expression plasmids for N-Bak, Bak, N-Bak-L76E and Bcl-xL are from InvivoGen; HeLa, primary glia and NIH-3T3 cells were grown in DMEM (Invitrogen) resulting in Bak-5′-UTR-Luc. In the presence of35S-methionine (Perkin-Elmer, Waltham, MA, USA) translation was performed in the rabbit reticulocyte lysate using TnT Quick Coupled Transcription/Translation System (Promega). The in vitro translation product was purified using TFN-Quick Coupled Transcription/Translation System (Promega) in the presence of35S-methionine (Perkin-Elmer, Waltham, MA, USA) according to the manufacturer’s instructions. HeLa cells were transfected by Lipofectamine 2000 (Invitrogen). Blockage of translation by CHX was optimized by metabolic labeling of SCG neurons followed by precipitation of the proteins by trichloro-acetic acid and measuring the radioactivity by scintillation beta-counter. Treatment with 20 μg/ml of CHX for 4 h blocked 90–95% of new protein translation.

**Primers.** PCR primers were designed using Primer Express v2.0. software (Applied Biosystems, Carlsbad, CA, USA) or OligoAnalyzer software (eudna.com) and ordered from Sigma. PCR primers were designed as published.6,36,38

**Biochemical methods.** In vitro translation was performed in the rabbit reticulocyte lysate using TFN-Quick Coupled Transcription/Translation System (Promega) in the presence of35S-methionine (Perkin-Elmer, Waltham, MA, USA) according to the manufacturer’s instructions. HeLa cells were transfected by Lipofectamine 2000 (Invitrogen). Blockage of translation by CHX was optimized by metabolic labeling of SCG neurons followed by precipitation of the proteins by trichloro-acetic acid and measuring the radioactivity by scintillation beta-counter. Treatment with 20 μg/ml of CHX for 4 h blocked 90–95% of new protein translation.

**Conflict of Interest**

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Disease website (http://www.nature.com/cddis)